p53 MEDIATED MESENCHYMAL-TO-ENDOTHELIAL TRANSITION IS A NOVEL MECHANISM OF VASCULOGENESIS AFTER ISCHEMIC CARDIAC INJURY

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ABSTRACT

ERIC SCOTT UBIL: p53 mediated mesenchymal-to-endothelial transition is a novel mechanism of vasculogenesis after ischemic cardiac injury
(Under the direction of Arjun Deb)

The mammalian heart displays limited regenerative capacity after acute ischemic injury and heals primarily through fibrosis. Recent therapeutic research has focused on increasing vasculature at the site of injury as a means of preserving remaining myocardium and improving cardiac function after injury. Other research has focused on the ability to reprogram cardiac fibroblasts using exogenous transcription factors to achieve a similar goal. However, the ability of cardiac fibroblasts to adopt alternate cellular fates in the absence of exogenous factors is unclear. Here, we demonstrate that a subset of cardiac fibroblasts adopts the physiological and anatomical characteristics of native endothelial cells after ischemic cardiac injury in the absence of any added factors. Using mice harboring genetically labeled fibroblasts (Col1a2-CreERT:R26R^{tdTomato}), we show that approximately 30% of labeled cardiac fibroblasts in the injury border zone express endothelial markers such as VE-cadherin, eNOS, Occludin, and Claudin 5. Fibroblast derived endothelial cells comprised 25+/-2% of total luminal endothelial cells at the border zone 3 days after injury. To better understand fibroblast-endothelial transition we subjected cardiac fibroblasts to cellular stress (serum starvation) and found that they formed endothelium-like structures on Matrigel and up-regulated endothelial specific genes (e.g. VE-cadherin, Flk1, Flt1) 6-20 fold. We show that transition of fibroblasts to endothelial-like cells *ex vivo* is p53 dependent. Pharmacological inhibition of p53 using Pifithrin-α or genetic deletion in fibroblasts (Col1a2-CreERT:p53$^{fl/fl}$) led to a 94% mean decrease in Matrigel tube formation and 90% reduction

in endothelial gene expression. Moreover, using semi-quantitative immunofluorescent staining, we observed that p53 levels in cardiac fibroblasts were more than 6 fold higher at the injury border zone. Injection of a p53 activator, RITA, after injury doubled p53 levels in cardiac fibroblasts and increased the degree of mesenchymal-to-endothelial transition (MEndoT) by 43%. Enhanced MEndoT was also associated with decreased collagen deposition and improved heart function 7 days post injury. In summary, we show that cardiac fibroblasts adopt endothelial cell like fates after cardiac injury and contribute to the neovascularization of the injured region. Manipulation of MEndoT could represent a novel therapeutic strategy to increase post infarct angiogenesis and enhance function in the injured heart.

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PREFACE

The work presented has been submitted for publication. My roles in this project included design, performance, and analysis of experiments and assistance in preparing the manuscript. Indu Pillai and Jinzhu Duan performed flow cytometry experiments and corresponding data analysis (Figure 2.1, Figure 2.2 and Figure 2.4). Francesca Bargiacchi performed the Western blot (Figure 3.4). Mauricio Rojas performed the animal surgeries and echocardiography as well as assisted with the corresponding data analysis. Arjun Deb was the principal investigator and helped design experiments, analyze data and write the manuscript.

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LIST OF ABBREVIATIONS

αSMA	alpha-Smooth Muscle Actin
AcLDL	Acetylated Low Density Lipoprotein
AV	Atrio-Ventricular
bFGF	basic Fibroblast Growth Factor
BMP7	Bone Morphogenic Protein 7
ChIP	Chromatin Immunoprecipitation
DiO	3,3'-Dilinoleyloxacarbocyanine
DLAM	Department of Laboratory Animal Medicine
EMT	Epithelial-to-Mesenchymal Transition
EndoMT	Endothelial-to-Mesenchymal Transition
eNOS	endothelial Nitric Oxide Synthase
EPDC	Epicardially Derived Cells
FBS	Fetal Bovine Serum
FN-ED-A	Fibronectin Extra Domain A splice variant
IACUC	Institutional Animal Care and Use Committee
IL-1	Interleukin 1
IL-6	Interleukin 6

IMDM	Iscove’s Minimal Defined Medium
IR	Ischemia Reperfusion
LAD	Left Anterior Descending
LIF	Leukemia Inhibitory Factor
MAPK	Mitogen Activated Protein Kinase
MEndoT	Mesenchymal-to-Endothelial Transition
MET	Mesenchymal-to-Epithelial Transition
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cell
NIH	National Institutes of Health
PDGF	Platelet Derived Growth Factor
RITA	Reactivator of p53 Induced Tumor cell Apoptosis
ROS	Reactive Oxygen Species
TAC	Trans-aortic Constriction
TAD	Trans-Activation Domain
TGF-α	Transforming Growth Factor alpha
TGF-β	Transforming Growth Factor beta
TIMP	Tissue Inhibitor of Metalloproteinase

TNF-α	Tumor Necrosis Factor alpha
VECAD	VE-cadherin
WGA	Wheat Germ Agglutinin
Wt1	Wilms' Tumor 1

CHAPTER 1 Introduction

Myocardial infarction and ischemia reperfusion injury

Myocardial infarction, also known as heart attack, is the leading cause of morbidity and mortality in the United States. Each year, approximately 635,000 patients are diagnosed as having experienced a first myocardial infarction with another 280,000 experiencing a recurrent infarction[1].

During myocardial infarction, coronary blood flow is occluded, leading to the death of dependent downstream ventricular myocardium. As the mammalian heart has limited regenerative capacity there is increased hemodynamic burden on the remaining cardiomyocytes, often leading to maladaptive remodeling in the heart and heart failure. The heart heals primarily through fibrosis and scarring which are largely mediated by the cardiac fibroblast. In the following sections an overview of cardiac fibroblasts and their roles in normal physiology and in the wound healing process will be described.

Overview of cardiac fibroblasts

Cardiac fibroblasts are the most populous type of cells in the heart and comprise 40-60% of non-myocyte cells within the adult rodent heart[2]. They are a highly heterogeneous population of cells that is spatially distributed throughout the heart[3,4]. Morphologically, fibroblasts have been characterized as flat, spindle-shaped cells with numerous processes emanating from the main cell body[5].

Cardiac fibroblasts reside within the myocardial interstitium and form a supportive stromal framework[6]. They are also highly migratory and tend to form only weak attachments to the basement membrane and to neighboring cells[7].

Gene expression within the fibroblast population varies and is partially determined by the local environment[7]. To date, a common expression marker has not been identified which can specifically label all of the subpopulations of cardiac fibroblasts. Instead, research has mainly relied on the use of semi-specific markers to differentiate fibroblasts from other cell types. As an example, expression of DDR2 has been commonly linked to cardiac fibroblasts though it is also expressed in fibrocytes and endothelial cells. Similarly, Fsp1 expression has been used to identify cardiac fibroblasts but has also been demonstrated in various cancer cells. Table 1.1 provides a summary of the most commonly used fibroblast markers and their expression in other cell types. Lack of a commonly expressed fibroblast marker has been a significant challenge in the study of cardiac fibroblasts.

Developmental origins of cardiac fibroblasts

Cardiac fibroblasts are derived predominantly from the developing epicardium[8,9]. Cells from the emergent proepicardium migrate across the surface of the embryonic heart forming the epicardium, a single cell thick epithelial sheet covering the heart. Cells derived from the epicardium, also known as Epicardially Derived cells (EPDCs), undergo Epithelial-to-Mesenchymal Transition (EMT)[10,11,12,13,14,15,16] in response to multiple growth factors such as PDGF, TGF, and FGF[17]. After adopting a mesenchymal phenotype, the nascent fibroblasts invade the developing myocardium, which is facilitated by their lack of stable cell-cell and cell-matrix adhesions.

Cardiac fibroblasts within the developing myocardium express extracellular matrix (ECM) proteins that govern the patterning of the developing heart. The appropriate deposition and organization of ECM in the developing heart is particularly critical for electrical insulation of the atria from the ventricles[18], in the patterning of the atrioventricular (AV) node[5] and the annulus fibrosis[18]. Within the endomysial network, fibroblasts also surround bundles of cardiomyocytes in a lamellar fashion and enable propagation of electrical activity along specific pathways[19,20,21].

Unlike fibroblasts that reside within the myocardial interstitium, valvular fibroblasts are derived from endothelial cells within the developing cardiac cushion through a process termed Endothelial-to-Mesenchymal Transition (EndoMT). In response to TGF-β, PDGF, and Wnt the endothelial cells delaminate, adopt a fibroblast phenotype and then invade the cardiac jelly and contribute to the formation of collagenous valve leaflets and the valve interstitium[22,23].

Fibroblast functions in normal physiological conditions

Cardiac fibroblasts are known to play two key roles in basal physiological conditions which will be described in the following sections. First, fibroblasts maintain the ECM framework of the heart. Secondly, fibroblasts help to regulate electrical circuits within the heart. Both functions are critical to the continuous regulated beating in the healthy heart.

Cardiac fibroblasts provide the structural framework of the heart

During basal heart function, cardiac fibroblasts provide the three dimensional extracellular matrix (ECM) scaffolding for the various cell types within the heart. The scaffold provides the structural framework of the heart, distributing mechanical force throughout the tissue and providing attachment points for the all the other cell types that reside there[5].

The ECM is maintained by cardiac fibroblasts through a homeostatic balance of synthesis and degradation of numerous collagens, glycoproteins, and proteoglycans[24,25]. One of the key components of the ECM is fibrillar collagen, which when cross-linked provides tensile strength. Roughly 90% of the collagens found in the heart are types I and III. To a lesser extent, fibroblasts also secrete collagen types IV, V, and VI as well as elastin and laminin[26].

To maintain homeostatic balance, fibroblasts also degrade the ECM by secretion of matrix metalloproteinases (MMPs). MMPs are a family of zinc dependent proteinases that degrade collagen and other components of the ECM. Fibroblasts can further control MMP mediated degradation of the ECM through the secretion of tissue inhibitors of metalloproteinases (TIMPs), which can effectively block the activity of MMPs[27].

Cardiac fibroblasts provide electrical insulation and electrical coupling of cardiomyocytes

In addition to providing the scaffold for the heart, the ECM also serves to insulate groups of myocytes, allowing electrical separation and differential contraction of the heart chambers. This property allows the regulated sequential beating of various parts of the heart, as seen in the electrical separation of the atria and ventricles by the annulus fibrosis, allowing asynchronous cardiac contraction[28].

A subset of cardiac fibroblasts has also been demonstrated to play a more direct role in the electrophysiology of the heart. Through gap junctions with myocytes, mediated by connexins 40, 43, and 45[29,30,31,32], fibroblasts can facilitate electrical signal transduction and link cardiomyocytes that would otherwise be electrically insulated by the ECM. Fibroblasts are non-excitable cells with high membrane resistance, which makes them good conductors of electrical signals[5].

Roles of fibroblasts after cardiac injury

After cardiac injury, the fibroblast is one of the primary effectors of fibrotic wound healing and scarring. As described in this section, the cardiac fibroblast communicates with other cell types to mediate the progression of wound healing in the context of the injured site.

Within the first 24 hours after cardiac injury, the injured region is infiltrated by a polymorphonuclear infiltrate. Over the next 48 hours inflammatory cells, such as monocytes and macrophages, replace the polymorphonuclear infiltrate and facilitate apoptosis and phagocytosis of necrotic cells[6,33]. Macrophages also express several growth factors, such as PDGF[34], TNFa[35], IL-1 and IL-6[36]; that induce proliferation and migration of resident fibroblasts as well as localization of circulating cells, which leads to an increase in the number of fibroblasts and myofibroblasts at the site of injury.

After injury, macrophages also secrete pro-angiogenic stimuli such as VEGF[37]. As the macrophage population declines, activated cardiac fibroblasts take over as key expressors of pro-angiogeneic cytokines and regulators of endothelial cell proliferation[38,39]. This pro-angiogenic effect of cardiac fibroblasts is an important repair response and leads to the formation of the fibroblast and endothelium rich granulation tissue, an early stage of the fibrotic scar.

During granulation tissue formation, fibroblasts continue to proliferate and migrate from other areas of the heart in response to chemotactic ques. They also increase collagen deposition at the injury border zone and progressively migrate towards the center of in the infarct site[33]. In the early stages of wound healing (i.e. within 3 days), fibroblasts primarily secrete Type III collagen but switch to Type I collagen as wound healing progresses[40]. Cross-linked Type I collagen confers tensile strength to the wound site which helps to

prevent excess infarct site expansion and reduces the chances of cardiac rupture from increased hemodynamic burden[41].

As wound healing progresses, there is increased activation of fibroblasts to adopt a myofibroblast phenotype. The contractile properties of the myofibroblast foster wound contraction and remodeling of the ECM to form the mature scar. ECM remodeling takes place partly through the degradation of collagen fibers, which occurs after a half-life of approximately 80-120 days and is mediated by zinc-dependent MMPs including collagenases and gelatinases[42].

During the remodeling process, fibroblasts and endothelial cells reach their peak numbers at approximately 7 days after injury[43] and then begin to decline as the scar matures, leaving a largely acellular scar[44]. In the mature scar, ECM can occupy as much as 80% of the infarct area[41]. The scar tends to persist, and populations of myofibroblasts which maintain the scar have been observed at the site of injury as long as 20 years later[45].

Fibroblast adoption of the myofibroblast phenotype after injury

Myofibroblasts are present after cardiac injury but not before[46]. They are a specialized cell type with the migratory and ECM secretory characteristics of fibroblasts but are also capable of exerting contractile forces. Myofibroblasts can be distinguished from fibroblasts in their expression of alpha-Smooth Muscle Actin (αSMA)[33]. Myofibroblasts also differ from smooth muscle cells, which express αSMA, by their fibroblast characteristics and by their relatively lower expression of smooth muscle myosin heavy chain[33].

After injury, myofibroblasts are derived from a number of diverse sources including resident fibroblasts, endothelial cells (through EndoMT)[47], epithelial cells (through EMT)[48], mesenchymal stem cells (MSCs)[49], smooth muscle cells[49], and pericytes[50,51]. Circulating

fibrocytes[52], which are a distinct population of leukocytes with characteristics of fibroblasts, and a subpopulation of monocytes that coexpress monocytic markers (i.e. CD45 and CD11b) and myofibroblast markers (i.e. Fsp1 and αSMA) have also been shown to contribute to the myofibroblast population[53]. The relative contribution of each source to the myofibroblast pool after injury varies by study and to date the relative contribution of each source has yet to be fully delineated.

The conversion of fibroblasts to myofibroblasts is thought to take place through an intermediate phenotype known as the proto-myofibroblast. Fibroblasts exposed to increased mechanical stress by post-injury disruption of the ECM framework or PDGF will adopt the intermediate state[46]. Proto-myofibroblasts can then be induced to adopt a myofibroblast fate by exposure to TGF-β or Fibronectin Extra Domain A splice variant (FN-ED-A)[46]. TGF-β is primarily secreted by fibroblasts, and provides chemotactic cues to migratory myofibroblasts, attracting them to the site of injury[33]. Inflammatory cells such as macrophages, T cells and mast cells have the ability to negatively regulate the effects of TGF-β activation by expression of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, and TGF-α), which can inhibit the adoption of the myofibroblast state[54].

After the adoption of the myofibroblast phenotype, the contractile properties of the cells facilitate the scar compaction typical in maturing scars. The contractility of myofibroblasts is partially regulated by factors such as TGF-β1, relaxin, and angiotensin II[5,33]. Interestingly, cardiomyocytes can effectively produce angiotensin and locally regulate myofibroblast contractility.

Scar compaction and remodeling of the ECM leads to the formation of a dense and well organized scar with increased tensile properties. The contractile actions of the myofibroblasts have also been proposed to slow adverse cardiac remodeling by preventing infarct expansion and ventricular dilation which would otherwise lead to wall thinning and

cardiomyocyte hypertrophy[33]. Interference with myofibroblast recruitment and contractility has been demonstrated to negatively affect cardiac remodeling after injury[33].

Endothelial-to-Mesenchymal Transition

The cardiac fibroblast serves a number of roles before injury and is essential to cardiac repair after injury. Endothelial-to-Mesenchymal Transition, or EndMT, has been described as a source of fibroblasts and myofibroblasts after injury. In a murine trans-aortic constriction (TAC) model of chronic high blood pressure, Zeisberg and colleagues used lineage tracing to determine that genetically labeled endothelial cells adopt a fibroblast phenotype in response to chronic injury[55]47. This novel finding demonstrated that cellular plasticity exists between endothelial cells and fibroblasts and has since been confirmed by several other groups[56,57,58,59,60].

Prior to injury, endothelial cells comprise the lining of the blood vessel lumen[61]. Morphologically, they are flat cells that use mechanosensory cues to align with directional blood flow[62]. Endothelial cells differ from fibroblasts in their ability to form strong intercellular junctions, such as adherens and tight junctions, which create a selective barrier to blood flow and preclude endothelial cells from having migratory traits[63].

Given the cellular plasticity that has been demonstrated by endothelial cells after chronic cardiac injury via EndMT, it led us to question whether ischemic cardiac injury could trigger fibroblasts to undergo the reverse process, which we have termed Mesenchymal-to-Endothelial Transition (MEndoT), and adopt an endothelial phenotype.

RESEARCH PRESENTED IN THIS DISSERTATION

As described in the following chapter summaries, the goals of this dissertation thesis are:

Chapter 2. To determine whether cardiac fibroblasts undergo MEndoT after injury

Angiogenesis and the restoration of blood flow to the ischemic area are essential to continued wound healing after cardiac injury. My data demonstrates that in addition to the previously described role of secretion of pro-angiogenic factors during scar formation, a subset of cardiac fibroblasts contribute directly to the pool of endothelial cells at the site of injury by adopting an endothelial phenotype. Further, I show that endothelial cells derived from cardiac fibroblasts are incorporated into the vascular network and are capable of supporting blood flow.

Chapter 3. To identify the signaling mechanisms that cause MEndoT to occur

Previous studies have shown that p53 is upregulated in the heart after ischemic injury. More recently studies have also shown a role for p53 in conversion of cellular phenotypes in processes such as EMT. To identify whether p53 plays a role in MEndoT we utilized an *ex vivo* serum starvation model system, which has been previously shown to increase p53 levels in cells, to determine whether cardiac fibroblasts could be induced to undergo MEndoT. After successfully demonstrating that labeled cardiac fibroblasts can adopt functional endothelial traits and upregulate endothelial-specific genes in the serum starvation model, we utilize p53-based gain and loss of function approaches to determine whether p53 plays a regulatory role in MEndoT. We found that *ex vivo* pharmacological inhibition or deletion of p53 during serum starvation reduced MEndoT while increasing p53 levels using a small molecule activator known as RITA increased MEndoT.

Chapter 4. To determine the functional role of MEndoT in cardiac repair

After identifying that p53 regulates MEndoT in an *ex vivo* model system, I describe how we utilized p53-based gain and loss of function approaches to determine the physiological role of MEndoT in cardiac repair. Using a p53 genetic deletion model, I show that in the absence of p53 in cardiac fibroblasts MEndoT is decreased, as is the cardiac vasculature at the site of injury. I also demonstrate that lack of MEndoT is associated with reduced cardiac function and increased scarring. Alternatively, increasing p53 levels in cardiac fibroblasts after cardiac injury through treatment with RITA led to an increase in MEndoT and an increase in cardiac vasculature at the site of injury. This increase in vascularity was associated with improved heart function and reduced scar formation.

Chapter 5. Conclusions and perspectives

In this chapter I summarize the findings demonstrated in this dissertation. I also describe some of the main limitations of the work and how they affect our current understanding of MEndoT. In addition, I describe some of the outstanding questions that could be addressed in the future study of MEndoT as well as potential implications in the treatment of human cardiac disease.

Supplemental Material

Table 1.1 Expression of Fibroblast Markers in Other Cell Types

Fibroblast Marker	Abbreviated Gene Name	Other Cell Types Expressing	References
alpha-Smooth Muscle Actin	αSMA	Cardiomyocytes, Mesenchymal stem cells, Myoepithelial cells, Pericytes, Smooth muscle cells	Potta et al. (2010), Chin et al. (2013), Katori et al. (2013), He et al. (1995), Kawasaki et al. (2008)
CD40	n/a	B cells, Carcinoma/Leukemia, Monocytes, Dendritic cells, Endothelial cells, Epithelial cells	Clark et al. (1990), van Kooten et al. (2000)
Collagen type I alpha 1	Col1a1	Chondroblasts, Osteoblasts	Perrier-Groult et al. (2013), Cleriques et al. (2012)
Collagen type I alpha 2	Col1a2	Osteoblasts	Duan et al. (2013)
Fibroblast specific protein-1	Fsp1 (or S100A4)	Carcinoma, Macrophages, Myoepithelial cells, Smooth muscle cells	Chao et al. (2012), Osterreicher et al. (2010), Katori et al. (2013), Sugimoto et al. (2006)
Thymus cell antigen-1	Thy-1 (or CD90)	Endothelial cells, Leukocytes	Tuffin et al. (2008), Vermiere et al. (2011)
Vimentin	Vim	Carcinoma, Endothelial cells, Myoepithelial cells, Pericytes, Smooth muscle cells	Chao et al. (2012), Conway et al. (2013), Beha et al. (2012), Kokovay et al. (2006), Kacem et al. (1995)

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CHAPTER 2 Cardiac fibroblasts undergo Mesenchymal-to-Endothelial Transition in response to acute cardiac injury

Overview

Objective: After acute cardiac injury there is a close and coordinated relationship between fibroblasts and endothelial cells during their proliferative stage in the early fibrotic response. It has been previously demonstrated that after cardiac injury a subset of endothelial cells adopts a fibroblast fate through the process of Endothelial-to-Mesenchymal Transition (EndMT). While cardiac fibroblasts are thought to be terminally differentiated cells, whether they can adopt an endothelial fate after injury is unknown. We therefore sought to determine whether fibroblasts undergo the reverse process, Mesenchymal-to-Endothelial Transition (MEndoT), after acute ischemic injury.

Methods and Results: We utilized a fate mapping model to determine whether cardiac fibroblasts undergo MEndoT after ischemia reperfusion cardiac injury. To do so, we utilized a tamoxifen inducible Cre recombinase driven by the fibroblast specific regulatory sequence of the alpha2 (Type 1) collagen gene (Col1a2-CreERT). Tamoxifen treated Collagen1a2-CreERT:R26R^{tdTomato} reporter mice underwent ischemia reperfusion or sham injury, then cardiac sections were prepared 3, 7, and 14 days after injury and immunostained for endothelial-specific markers. We observed that approximately 30% of labeled cardiac fibroblasts expressed endothelial markers after ischemia reperfusion injury but did not in sham injured animals. We also utilized DiO perfusion and AcLDL uptake assays and found

that MEndoT cells are integrated into the vasculature and have functional traits of endothelial cells after injury.

Conclusions: We demonstrate that after acute cardiac injury, a subset of cardiac fibroblasts adopt an endothelial fate through a process we have termed MEndoT. We also demonstrate that MEndoT cells integrate with the vascular network and support blood flow at the site of injury.

Introduction

Recent research has demonstrated that fibroblasts and endothelial cells possess cellular plasticity that is activated after cardiac injury. Zeisberg and colleagues showed that a subset of resident endothelial cells adopt a fibroblast phenotype through the process known as Endothelial-to-Mesenchymal Transition (EndMT)[1] in response to cardiac injury. From a therapeutic standpoint, the inhibition of EndMT led to reduced fibrosis and the negative cardiac remodeling associated with chronic pressure overload injury and suggests that manipulation of cellular plasticity could be a novel treatment for cardiac injury.

That led us to question whether in an ischemic model of cardiac injury if it was possible for fibroblasts to undergo the process of Mesenchymal-to-Endothelial Transition (MEndoT) and contribute to the pool of endothelial cells that restore the vasculature at the site of injury.

Materials and Methods

Generation of transgenic mice

All animal experiments were conducted in accordance with the University of North Carolina Institutional Animal Care and Use Committee (IACUC) guidelines. Collagen1a2-CreERT:R26R^{tdTomato} mice were obtained by breeding Collagen1a2-CreERT mice[2,3] with R26R^{tdTomato} mice[4]. Genetic labeling was achieved by injecting 8-10 week old Collagen1a2-CreERT:R26R^{tdTomato} mice with1mg tamoxifen (Sigma) intraperitoneally daily for 10 days. 5 days after cessation of tamoxifen the mice either underwent ischemia reperfusion or sham injury or cardiac fibroblasts were isolated.

Cardiac injury model

Mice were anesthetized with 3% isoflurane (Butler Schein), maintained at 2% isoflurane and intubated using a Harvard Rodent Volume-Cycled Ventilator. Ischemia-reperfusion injury was induced by ligation of the left anterior descending (LAD) coronary artery for 30 minutes followed by reperfusion[5]. Sham injury was performed using the same procedure but the ligature was not tightened to occlude blood flow. At 3, 7, or 14 days the injured animals were anesthetized with pentobarbital (45mg/kg) and the left ventricle perfused with 5ml PBS (Gibco) followed by 2ml 4% paraformaldehyde (Electron Microscopy Sciences). For DiO staining of cardiac vasculature, DiO was prepared as described[6] and 2 ml of 120μg/ml DiO was perfused prior to PFA perfusion. For AcLDL staining, 1μg Alexa488 conjugated AcLDL was injected via jugular vein catheter and the heart harvested as described 4 hours later. After perfusion, the heart was isolated and fixed for 1 hour in 4% paraformaldehyde at 4^{o}C. Then the heart was fixed for an addition 4 hours in new 4% paraformaldehyde solution at 4^{o}C. Hearts were then sucrosed embedded overnight at 4^{o}C in a 30% sucrose (MP Biochemicals) solution and frozen in OCT (Tissue-Tek).

Immunofluorescent staining , confocal imaging, and quantitation

Frozen sections (7µm thick) were prepared from hearts isolated 3, 7, or 14 days after ischemia reperfusion or sham injury of tamoxifen treated Col1a2CreERT:R26R^{tdTomato} mice. Frozen sections were also prepared from uninjured tamoxifen treated Col1a2CreERT:R26R^{tdTomato} mice. Prepared sections were permeabilized for 10 minutes in acetone at -20^{o}C, washed with PBS, and blocked with 10% normal goat serum with 1% BSA. Staining was subsequently carried out using a primary antibody to VECAD, eNOS, Claudin 5, Occludin, alpha-SMA, or CD68 (Abcam) and an associated fluorescently conjugated secondary antibody (Millipore). Table 2.1 outlines specific information for each antibody used. WGA staining of frozen sections from ischemia-reperfusion injured mice was performed as described[7]. DAPI stain was incorporated to visualize nuclei. Imaging of 5 non-overlapping areas of the injury border zone or areas distal to the border zone was performed for sections from each animal using the Lieca SP2 AOBS Upright Laser Scanning Microscope (Leica). Quantitation of colocalization was performed using ImageJ software (NIH). To determine the percentage of cells undergoing MEndoT, the number of tdTomato labeled cells colocalized with an endothelial marker were divided by the total number of tdTomato labeled cells. For determination of luminal surface area occupied by tdTomato cells the Manders coefficient of colocalization was determined using the JACoP[8] plugin for ImageJ.

Fibroblast isolation and culture

Cardiac fibroblasts were isolated from uninjured tamoxifen treated Col1a2-CreERT:R26R^{tdTomato} mice. At 8-10 weeks of age, hearts were isolated from euthanized mice. After washing 3 times with 1X HBSS (Gibco) the hearts were minced with a razor into approximately 1mm^2 pieces and digested using 5ml of a 0.1% Trypsin solution (Gibco) with 50U/ml Collagenase II (Worthington). 5 sequential digestions were performed and the supernatant filtered through a 40µm strainer and the trypsin neutralized with Fetal Bovine

Serum (FBS). Cells were cultured for 1 hour at 37^{o}C in IMDM, 1X Penicillin/Streptomycin, 10% FBS (Gibco). After 1 hour the medium was changed to F12K, 1X Penicillin/Streptomycin, 10% FBS (Gibco), 10ng/ml Leukemia Inhibitory Factor (LIF) (Millipore), and 10ng/ml bFGF (Millipore). Cells were grown at 37^{o}C, 5% CO_2 for 7-10 days until they became confluent.

Flow cytometry

Analytical flow cytometry was performed for cell surface markers using fluorophore conjugated antibodies to Thy1, CD31, VECAD, and c-Kit (eBioscience). Table 2.1 outlines specific information for each antibody. Cultured cardiac fibroblasts were dissociated using Accutase (Innovative Cell Technologies, Inc.) and immunostained in 0.1% BSA in PBS. Labeling was conducted at 1x10^{6} cells/ml for 20-30 minutes at 4^{o}C, followed by 2 washes with 0.1% BSA in PBS, and subsequent analysis on a Beckman-Coulter CyAn ADP instrument with Summit software (Dako).

Results

To determine whether cardiac fibroblasts adopt an endothelial fate after cardiac injury, we utilized a genetic fate mapping strategy based on the use of Cre/Lox technology. To enable our strategy we crossed mice harboring a tamoxifen inducible Cre recombinase driven by the fibroblast specific regulatory sequence of the alpha2 (Type 1) collagen gene (Col1a2-CreERT) with the lineage reporter strain R26R^{tdTomato}. When the mice were bred together we obtained Col1a2-CreERT:R26R^{tdTomato} mice. When these mice were injected with tamoxifen, the Cre recombinase became activated and led to the indelible labeling of cells expressing Col1a2 and their progeny.

To determine the efficiency of tdTomato fluorescent labeling in our fate mapping model, we injected Col1a2-CreERT:R26R^{tdTomato} mice with tamoxifen for 10 days starting at 8-10 weeks of age. Cardiac fibroblasts were harvested by differential attachment[9] 5 days after the cessation of tamoxifen. Using flow cytometry, we observed that 70% of cardiac fibroblasts isolated were tdTomato positive (Figure 2.1a). While there are no completely specific markers for fibroblasts, Thy1 is known to be robustly expressed in cardiac fibroblasts[10]. We observed that 71.2% of Thy1 positive cells also expressed tdTomato (Figure 2.1b).

We also sought to determine whether tdTomato labeling was specific to fibroblasts. To do so, we isolated cardiac fibroblasts from tamoxifen treated uninjured Col1a2-CreERT:R26R^{tdTomato} mice and analyzed them for the expression of the endothelial markers VECAD and CD31. We observed that 99.9±0.06% and 99.8±0.02% (mean±S.E.M.) of labeled cells did not express VECAD and CD31, respectively (Figure 2.2). To further determine the specificity of labeling, we immunostained cardiac sections prepared from tamoxifen treated uninjured Col1a2-CreERT:R26R^{tdTomato} mice. We observed that 99.4% and 100% of tdTomato labeled cells did not express αSMA and the macrophage marker CD68, respectively (Figure 2.3). We have previously shown that in this reporter mouse cardiomyocytes are not labeled[11]. We also assayed whether tdTomato labeled cells expressed c-Kit and found that 99.98±0.02% (mean±S.E.M.) were negative (Figure 2.4). Taken together, our results demonstrate that labeling of cardiac fibroblasts in our system is both specific and efficient.

Prior to determining whether cardiac injury could induce fibroblasts to adopt an endothelial phenotype we sought to confirm that Cre recombinase is not activated during cardiac injury. To do so we treated Col1a2-CreERT:R26R^{tdTomato} mice with corn oil, the vehicle used during tamoxifen injections, and subjected the mice to ischemia reperfusion

cardiac injury. 3 days after injury the hearts were harvested, sectioned, and immunostained with Alexa488 conjugated wheat germ agglutinin (WGA) to label plasma membranes as a counterstain. We observed that of the approximately 38,000 cells counted, only 28 were tdTomato labeled (0.07%) (Figure 2.5).

After validating our fate mapping model, we sought to determine whether cardiac injury would induce labeled cardiac fibroblasts to adopt an endothelial fate. We subjected tamoxifen treated Col1a2-CreERT:R26R^{tdTomato} mice to either sham or ischemia reperfusion injury 5 days after the cessation of tamoxifen. Hearts were harvested 3 days after injury and immunostained for the endothelial specific markers VECAD, eNOS, Claudin 5 and Occludin. We observed that endothelial markers were expressed in labeled fibroblasts of ischemia reperfusion injured hearts (Figure 2.6b) but not in sham injured hearts (Figure 2.6a). We quantitated the percentage of labeled fibroblasts cells expressing VECAD, eNOS, Claudin 5, and Occludin and determined that 35±3%, 24.4±4%, 44±4%, 35±3% (mean±S.E.M.) of tdTomato labeled cells expressed each marker, respectively (Figure 2.6c). Alternatively, in sham injured animals VECAD, eNOS, Claudin 5, and Occludin were expressed in 0.3%, 1%, 2%, and 2% of labeled fibroblasts, respectively (Figure 2.6c). We also observed that the number of endothelial marker expressing cardiac fibroblasts was relatively higher at the injury border zone and was reduced by approximately 60% at sites distal to the injury (Figure 2.6c). We next sought to determine the temporal expression of the endothelial marker VECAD in labeled fibroblasts. We observed that the percentage of labeled fibroblasts undergoing MEndoT was similar at 3, 7, and 14 days after injury (Figure 2.6d).

After determining that cardiac fibroblasts express endothelial markers, we assayed whether MEndoT cells are incorporated into the vasculature at the site of injury. To do so, we perfused sham and ischemia reperfusion injured Col1a2-CreERT:R26R^{tdTomato} mice with the lipophilic dye DiO. When perfused, DiO will incorporate into the plasma membranes of

cells lining the lumen of blood vessels. The dye then migrates within the plasma membrane of the exposed cells but will not move from cell to cell[6]. At 3, 7, and 14 days after injury, we perfused the vasculature with DiO and observed that in sham injured hearts <1% of the luminal surface area was occupied by tdTomato labeled cardiac fibroblasts (Figure 2.7e,j). However, after ischemia reperfusion injury, at 3, 7, and 14 days after injury 19±3%, 34±6%, and 36±6% (mean±S.E.M.) of luminal surface area was derived from labeled cardiac fibroblasts, respectively (Figure 2.7f-j).

In addition to DiO perfusion, we also tested whether labeled cardiac fibroblasts could take up AcLDL, which is characteristic of endothelial cells[12]. Three days after injury of tamoxifen treated Col1a2-CreERT:R26R^{tdTomato} mice, Alexa488 conjugated AcLDL was injected via jugular catheter. After 4 hours the hearts were harvested and sectioned. We observed that in sham injured hearts, labeled fibroblasts did not take up AcLDL (Figure 2.8a) but after injury they did (Figure 2.8b).

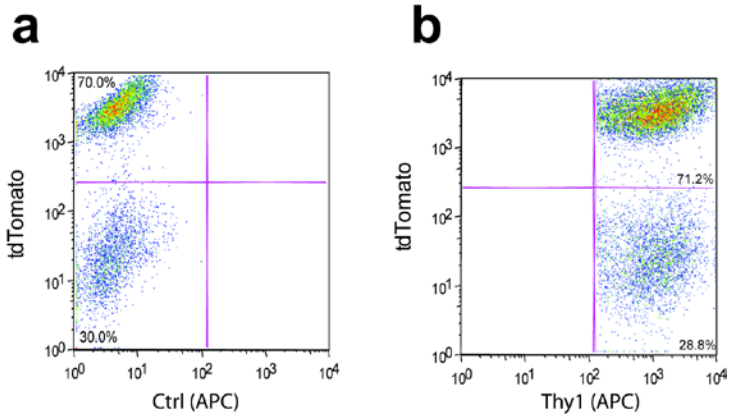
As previously described, after cardiac injury fibroblasts can adopt a myofibroblast phenotype. We sought to determine whether the sub-population of fibroblasts undergoing MEndoT is separate from those adopting the myofibroblast phenotype. To answer this question we immunostained for αSMA, which is characteristic of myofibroblasts, and VECAD in cardiac sections prepared 3 days after ischemia reperfusion injury in Col1a2-CreERT:R26R^{tdTomato} mice. We observed that αSMA expressing fibroblasts did not express VECAD (Figure 2.9), suggesting that the two populations are distinct.

Discussion

To the best of our knowledge, the process of MEndoT has not been previously described either during development or in any organ system after injury. Our finding is also novel because cardiac fibroblasts are thought to be terminally differentiated cells that are unable to adopt alternative phenotypes[13,14]. Recent work has focused on reprogramming cardiac fibroblasts to adopt other fates using exogenous transcription factors[15,16]. Our findings demonstrate that cardiac fibroblasts have a degree of native plasticity and that a subset of fibroblasts adopts an endothelial phenotype after injury in the absence of added exogenous factors.

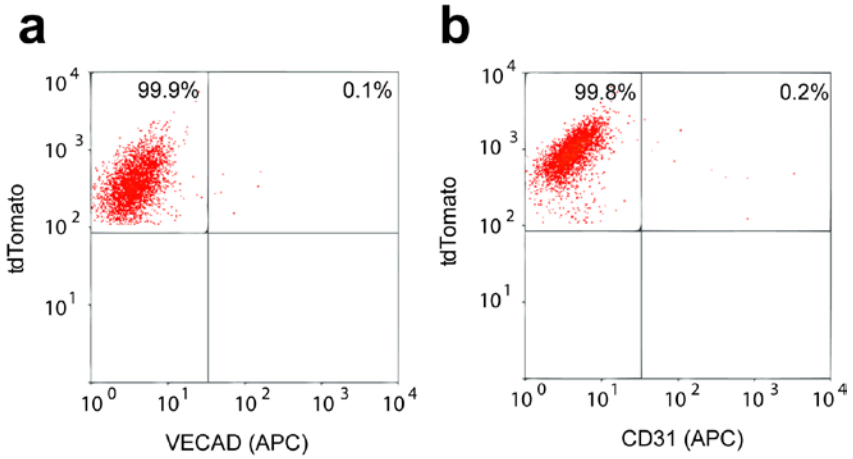
Our findings also show that in addition to regulating angiogenesis after injury, fibroblasts can provide a robust source of endothelial cells at the site of ischemic, directly contribute to the revascularization of the ischemic region and potentially enhance cardiac repair.

Figure 2.1 Labeling of cardiac fibroblasts in Col1a2-CreERT:R26R^{tdTomato} mice is efficient.



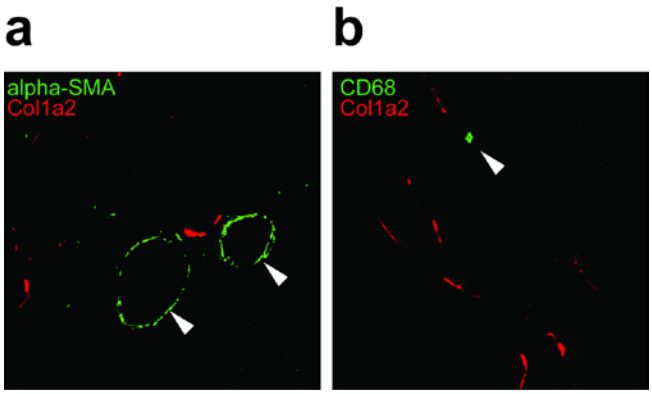
(a-b) Cardiac fibroblasts were isolated from uninjured 8-10 week old Col1a2-CreERT:R26R^{tdTomato} mice after 10 days of intraperitoneal tamoxifen injection. **(a)** Harvested cardiac fibroblasts were isolated by differential attachment and flow cytometry was utilized to determine the percentage of tdTomato labeled cells. **(b)** The percentage Thy1 positive cells expressing the tdTomato label was determined by flow cytometry.

Figure 2.2 Col1a2-CreERT:R26R^{tdTomato} labeled cardiac fibroblasts do not express endothelial specific markers.



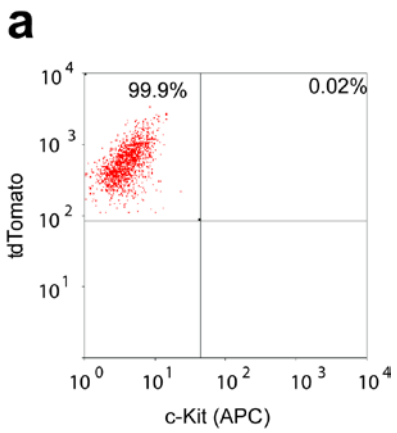
(a-b) Cardiac fibroblasts were isolated from uninjured 8-10 week old tamoxifen injected Col1a2-CreERT:R26R^{tdTomato} mice and analyzed by flow cytometry for the expression of **(a)** VECAD or **(b)** CD31.

Figure 2.3 Labeling of cardiac fibroblasts in Col1a2-CreERT:R26R^{tdTomato} mice is specific.



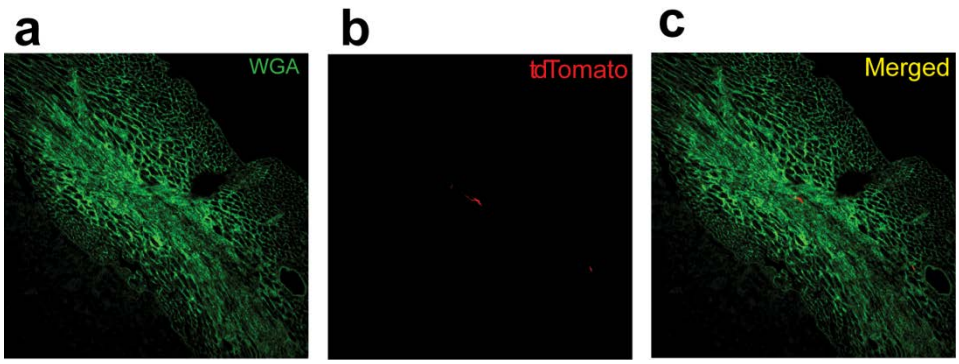
(a-b) Frozen heart sections were prepared from uninjured 8-10 week old tamoxifen injected Col1a2-CreERT:R26R^{tdTomato} mice and immunostained for **(a)** alpha Smooth Muscle Actin or **(b)** the macrophage marker CD68.

Figure 2.4 Col1a2-CreERT:R26R^{tdTomato} labeled cardiac fibroblasts are not positive for the stem cell marker c-Kit.



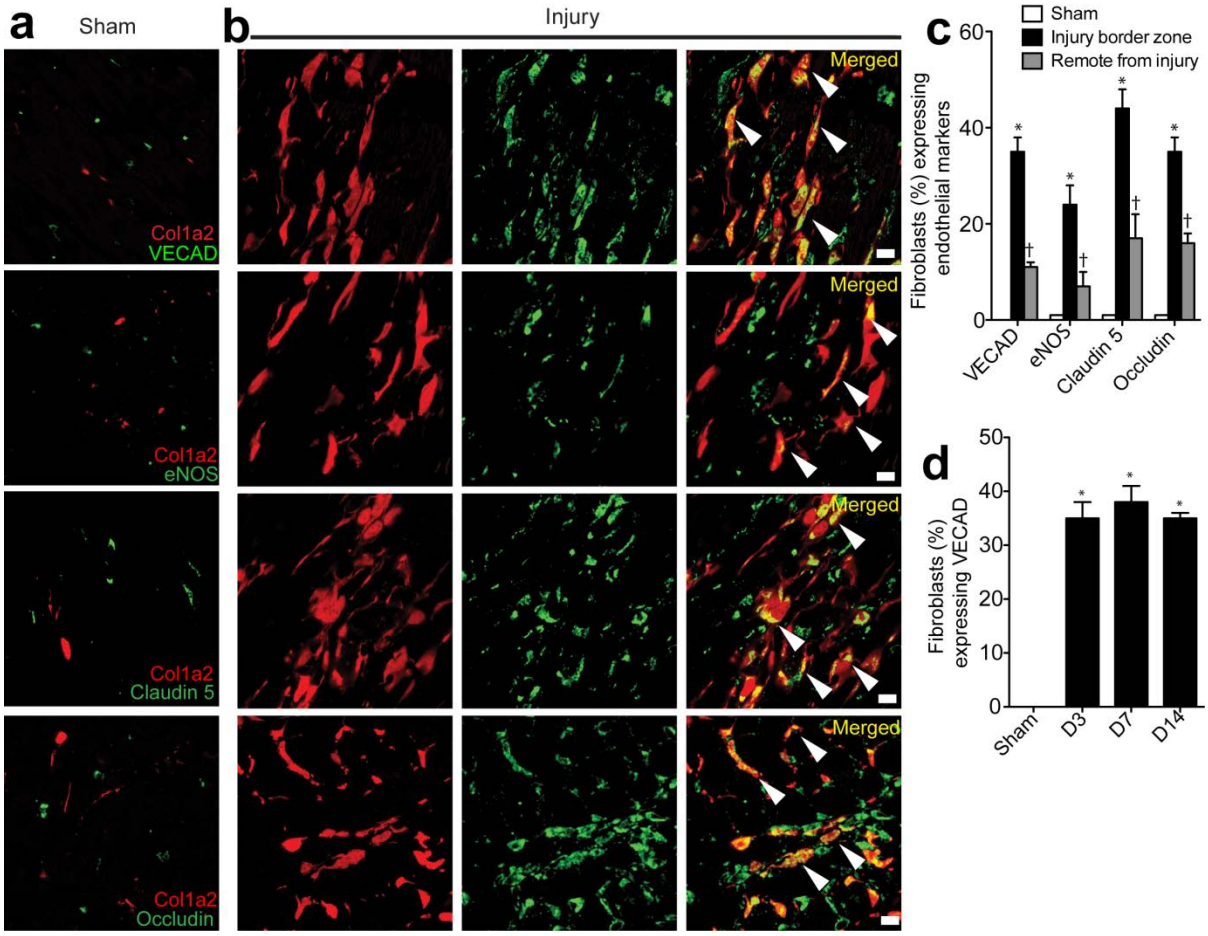
(a) Cardiac fibroblasts were isolated from uninjured 8-10 week old tamoxifen injected Col1a2-CreERT:R26R^{tdTomato} mice and analyzed by flow cytometry for the expression of c-Kit.

Figure 2.5 Cre recombinase is not activated by ischemia reperfusion cardiac injury.



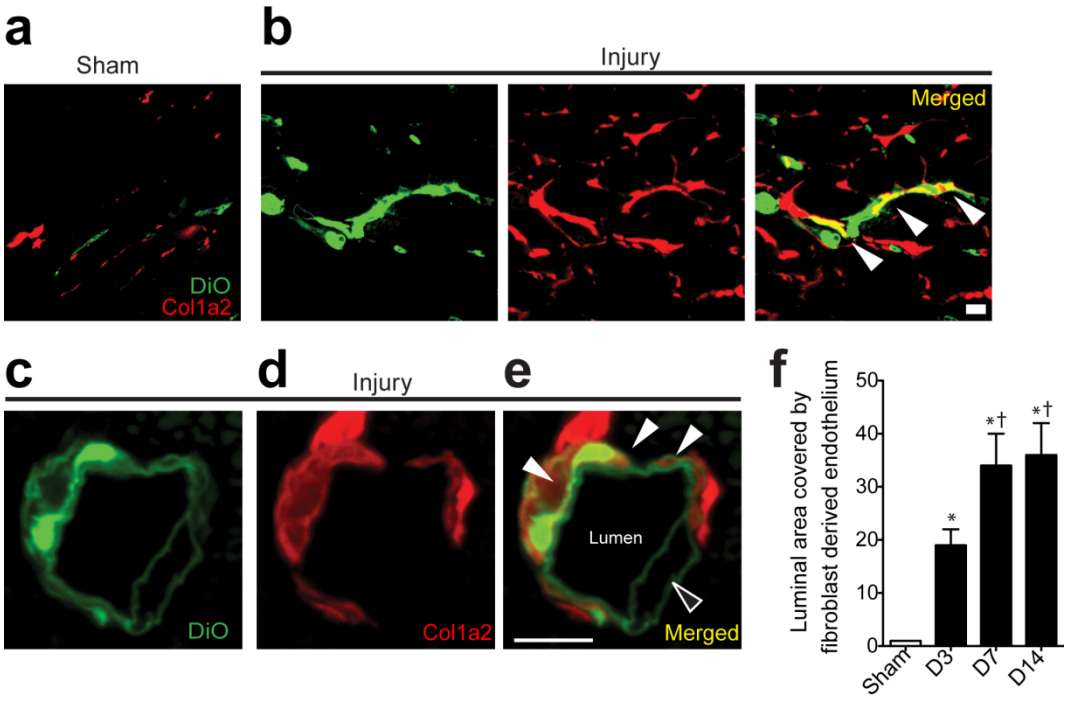
(a-c) Frozen heart sections were prepared from ischemia reperfusion injured 8-10 week old tamoxifen injected Col1a2-CreERT:R26R^{tdTomato} mice three days after injury. Sections were stained with **(a)** Alexa488 conjugated wheat germ agglutinin (WGA) to stain plasma membranes. **(b-c)** Visualization of **(b)** tdTomato labeled cells and colocalization in **(c)** merged image show rare labeled cells (28 labeled cells out of 38,000 counted (0.07%), n=3 animals).

Figure 2.6 Cardiac fibroblasts express endothelial markers after acute cardiac injury.



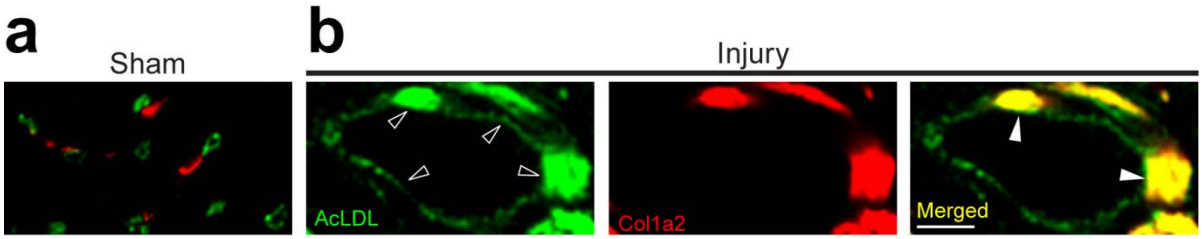
(a-b) Tamoxifen treated 8-10 week old Col1a2-CreERT:R26R^{tdTomato} mice were subjected to **(a)** sham or **(b)** ischemia reperfusion injury. Hearts were harvested 3 days after injury, sectioned and immunostained for VECAD, eNOS, Claudin 5, and Occludin. Colocalization is indicated by arrowheads (n=3 animals, scale bar = 10μm). **(c)** Percentage of cardiac fibroblasts expressing endothelial markers at the border zone and distal to the site of injury (*p<0.005 compared to sham except for eNOS and Claudin 5 which are p<0.05; †p<0.005 compared to injury border zone except for eNOS and Claudin 5 which are p<0.05; n=3 animals). **(d)** Temporal expression of VECAD by cardiac fibroblasts at the injury border zone (*p<0.005 compared to sham, n=3 animals per time point).

Figure 2.7 Cardiac fibroblasts occupy a luminal position after injury.



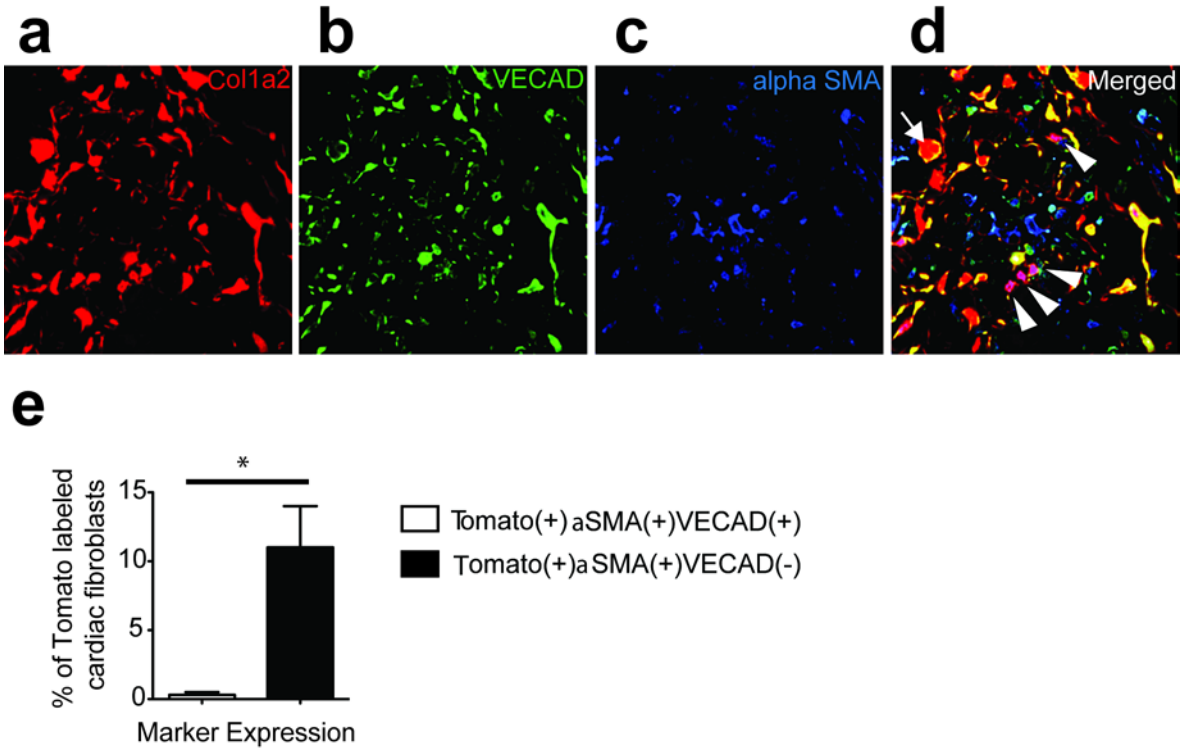
(a-e) Tamoxifen treated 8-10 week old Col1a2-CreERT:R26R^{tdTomato} were subjected to **(a)** sham or **(b-e)** ischemia reperfusion injury and hearts were DiO perfused and harvested 3 days after injury. **(b)** Longitudinal and **(c-e)** transverse sections of blood vessels in which labeled cardiac fibroblasts are DiO stained (arrowheads indicate fluorophore colocalization, open arrowhead indicates a DiO labeled endothelial cell; scale bar in **(b)** = 10μm, scale bar in **(e)** = 5μm; n=3 animals). **(f)** Quantitation of the percentage of the luminal endothelial surface derived from labeled cardiac fibroblasts at 3, 7, and 14 days after injury (*p<0.005 compared to sham, †p<0.05 compared to Day 3, n=3 animals at each time point).

Figure 2.8 Cardiac fibroblasts take up Acetylated LDL after acute cardiac injury.



(a-b) Tamoxifen treated 8-10 week old Col1a2-CreERT:R26R^{tdTomato} mice were subjected to **(a)** sham or **(b)** ischemia reperfusion injury, 3 days later Alexa488 conjugated AcLDL was injected, and after 4 hours the heart was harvested (open arrowheads indicate cells taking up AcLDL, arrowheads indicate colocalized fluorophores, scale bar = 5μm, n=3 animals).

Figure 2.9 Cardiac fibroblasts that undergo MEndoT do not express alpha Smooth Muscle Actin.



(a-d) Tamoxifen treated 8-10 week old Col1a2-CreERT:R26R^{tdTomato} mice were subjected to ischemia reperfusion injury and hearts were isolated 3 days later. Hearts sections show **(a)** labeled cardiac fibroblasts, **(b)** VECAD expressing cells, **(c)** alpha Smooth Muscle Actin expressing cells, and **(d)** colocalization of labeled fibroblasts and alpha Smooth Muscle Actin are indicated by arrowheads and colocalization of labeled fibroblasts and VECAD indicated by an arrow (n=3 animals). **(e)** Percentage of labeled cardiac fibroblasts expressing alpha Smooth Muscle Actin and VECAD (*p<0.005).

Supplemental Material

Table 2.1 Antibody Information

Application	Antibody	Vendor	Catalog Number
Immunostaining	VECAD	Abcam	ab33168
	eNOS	Abcam	ab66127
	Claudin 5	Abcam	ab53765
	Occludin	Abcam	ab31721
	αSMA	Abcam	ab5694
	CD68	Abcam	ab125212
Flow cytometry	Thy1	eBioscience	17-0902-81
	VECAD	eBioscience	17-1441-80
	CD31	eBioscience	17-0311
	c-Kit	BD Bioscience	561074

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CHAPTER 3 p53 Mediates MEndoT *ex vivo*

Overview

Objective: As a result of ischemia reperfusion injury, cells downstream of the occlusion are exposed to multiple forms of cellular stress such as hypoxia, nutrient deprivation and ROS. The MAPK pathway and p53 have been previously described as mediators of cellular stress in response to each of the aforementioned stressors. As p53 has been demonstrated to play a role in cell phenotype conversion, in this study we sought to determine whether p53 plays a role in the regulation of MEndoT.

Methods and Results: We demonstrate that p53 is upregulated in cardiac fibroblasts at the injury border zone after ischemia reperfusion injury. We also demonstrate that cardiac fibroblasts subjected to serum starvation *ex vivo* upregulate p53. Using multiple functional assays in our *ex vivo* model, we show that serum starvation can induce labeled cardiac fibroblasts to adopt endothelial traits in a p53 dependent manner. Inhibition of p53 signaling by pharmacological or genetic means disrupted MEndoT. Using a small molecule, RITA, to activate p53 we also observed that MEndoT in our *ex vivo* model could be increased.

Conclusions: Taken together, our results demonstrate that MEndoT following cellular stress occurs in a p53 dependent manner.

Introduction

Following ischemic cardiac injury, cells downstream of the coronary occlusion are exposed to stress caused by the disruption of blood flow. The two main forms of cellular stress in ischemic regions are hypoxia and nutrient deprivation as the flow of oxygen and nutrients normally delivered by the blood are abruptly reduced[1]. Both forms of stress contribute to the death of dependent myocardium and trigger the wound healing response described earlier. In the event that blood flow is restored, as would be the case during medical intervention, reperfusion injury can also occur. In this case reactive oxygen species (ROS) are generated as a byproduct of altered post-ischemic cellular metabolism. The ROS reduce the integrity of the surrounding cellular membranes and can lead to mitochondria mediated apoptosis[2,3].

The three aforementioned forms of stress have been previously demonstrated to elicit the cellular stress response through the Mitogen Activated Protein Kinase (MAPK) signaling pathway in the ischemia reperfusion injured heart [4,5]. As shown in Figure 3.1, one of the key effectors of the MAPK pathway is p53. p53 is evolutionarily conserved[6,7] and has been dubbed the "guardian of the genome"[8] for its roles in regulating cell cycle progression and the DNA damage response[9]. If the genome becomes compromised after DNA damage p53 holds the cell cycle in check until the DNA can be repaired. If the DNA cannot be repaired, p53 is a key initiator of the apoptotic pathway.

In recent studies, p53 has also been found to play conflicting roles in cell differentiation and reprogramming. Kawamura and colleagues determined that reduction in p53 led to improvements in the efficiency of generating iPSCs[10]. Alternatively, Molchadsky and colleagues discovered that context dependent cellular differentiation of mesenchymal progenitor cells was based on the regulation of p53. In their studies, Molchadsky and

colleagues determined that p53 inhibits differentiation into osteogenic, adipogenic, and myofibroblast phenotypes while p53 positively regulates the differentiation into skeletal muscle cells[11].

In two other studies Kim *et al* and Chang *et al* reported that p53 suppresses epithelial-to-mesenchymal transition (EMT) [12,13]. Additionally, Chang and colleagues demonstrated that when miR-200c, which is positively regulated by p53 activity, was restored after p53 deletion the cells reverted to a mesenchymal phenotype through the process of mesenchymal-to-epithelial transition (MET).

Extensive study of p53 has elucidated the protein structure and how it interacts with DNA (Figure 3.2)[14]. These findings have allowed researchers to develop pharmacological tools to manipulate p53 activity. As an example, an inhibitor of p53 activity called Pifithrin-α has been developed and can effectively block the DNA binding domain of p53[15,16]. To some extent, reduction of p53 mediated apoptosis using Pifithrin-α has been studied as a treatment for cardiomyocyte preservation after myocardial infarction[17,18,19,20]. Unfortunately, to date the studies have shown limited ability to improve overall wound healing.

Conversely, researchers have also discovered a means for positively regulating p53 activity based on the manipulation of the interaction between the p53 trans-activation domain (TAD) and MDM2, an E3 poly-ubiquitin ligase that adds a series of ubiquitins and thereby targets p53 for proteosomal degradation. In the event that the interaction between p53 and MDM2 is blocked, as is the case in the presence of the small molecule Reactivator of p53 Induced Tumor cell Apoptosis (RITA), p53 levels within the cell increase because proteosomal degradation is inhibited, which ultimately leads to cell cycle arrest or apoptosis. Applications of high doses of RITA have been studied as an approach to inducing apoptosis in cancerous cells[21,22,23,24].

Given that the activity of p53 was able to cause MET, we hypothesized that injury induced activation of p53 may similarly induce mesenchymal-to-endothelial transition (MEndoT). Utilizing our genetically labeled fibroblasts we developed an *ex vivo* model system that mimics post-ischemic injury induced cellular stress and recapitulates the MEndoT seen *in vivo*. Further, by manipulating p53 activity both positively and negatively, using RITA and Pifithrin-α respectively, we demonstrate that MEndoT is mediated by p53 activity.

Materials and Methods

Immunofluorescent staining of heart sections and relative p53 protein level determination

Frozen sections (7μm thick) were prepared from hearts isolated 3 days after injury from ischemia reperfusion or sham injured tamoxifen treated Col1a2-CreERT:R26R^{tdTomato} mice. Sections were permeabilized for 10 minutes in acetone at -20^{o}C, washed with PBS, and blocked with 10% normal goat serum with 1% BSA. Staining was subsequently carried out using a primary antibody to p53 (Abcam) and an associated fluorescently conjugated secondary antibody (Millipore). DAPI stain was incorporated to visualize nuclei. Imaging was performed using the Lieca SP2 AOBS Upright Laser Scanning Microscope (Leica). p53 localization in labeled cardiac fibroblasts was determined using the Manders coefficient as determined by the JACoP plugin of Image J software (NIH).

Fibroblast isolation and culture

Cardiac fibroblasts were isolated from uninjured tamoxifen treated Col1a2-CreERT:R26R^{tdTomato}, Col1a2-CreERT:p53$^{fl/fl}$ [25] or C57B/6 mice. At 8 weeks of age, hearts were isolated from euthanized mice. After washing 3 times with 1X HBSS (Gibco) the hearts were minced with a razor to approximately 1mm^2 pieces and digested using 5ml of a

0.1% Trypsin solution (Gibco) with 50U/ml Collagenase II (Worthington). 5 sequential digestions were performed and the supernatant filtered through a 40µm strainer and trypsin neutralized with Fetal Bovine Serum (FBS). Cells were cultured for 1 hour at 37^{o}C in IMDM, 1X Penicillin/Streptomycin, 10% FBS (Gibco). After 1 hour the medium was changed to F12K, 1X Penicillin/Streptomycin, 10% FBS (Gibco), 10ng/ml Leukemia Inhibitory Factor (LIF) (Millipore), and 10ng/ml bFGF (Millipore). Cells were grown at 37^{o}C, 5% CO_2 for 7-10 days until they became confluent.

Western blotting

Protein was isolated from cardiac fibroblasts cultured for 48 hours at 37^{o}C, 5% CO_2 in the presence or absence of FBS (IMDM, 1X Penicillin/Streptomycin, ±10% FBS). Isolated protein was concentration normalized, prepared with an SDS loading buffer, and run on a 12X Mini-PROTEAN TGX gel (BioRad) at 300V for 25 minutes. The protein was transferred to a nitrocellulose membrane using the Trans-Blot Turbo System (BioRad). The membrane was blocked using TBST+3% cold fish gelatin and probed using antibodies to p53 (Abcam) and alpha-Tubulin (Sigma). After washing, the bound primary antibodies were labeled with fluorescently conjugated secondary antibodies (LI-COR Biosciences). After washing with TBST the probed membranes were visualized on an Odyssey scanner (LI-COR Biosciences). Densitometry analysis was performed using ImageJ software (NIH).

Matrigel assay

Labeled fibroblasts (Col1a2-CreERT:R26R^{tdTomato}), p53 deleted fibroblasts (Col1a2-CreERT:p53$^{fl/fl}$), or C57B/6 fibroblasts were seeded on a Matrigel coated well at a density of 6X10^{4} cells per cm^{2} and cultured for 24 hours at 37^{o}C, 5% CO_2. Cells were grown in the presence or absence of FBS (IMDM, 1X Penicillin/Streptomycin, ±10% FBS). During pharmacological treatment studies, medium was supplemented with 100µM Pifithrin-α or 0.1µM RITA. The length of endothelial tube-like structures was quantitated using ImageJ software (NIH).

AcLDL uptake

Fibroblasts cultured for 48 hours in the presence or absence of serum (IMDM, 1X Penicillin/Streptomycin, ±10% FBS) were incubated with Alexa488 conjugated acetylated low density lipoprotein (AcLDL) (Invitrogen) for 4 hours at 37^{o}C, 5% CO_2. Uptake was visualized using the Lieca SP2 AOBS Upright Laser Scanning Microscope (Leica) either in the XY or XZ plane.

qRT-PCR

C57B/6 fibroblasts, or fibroblasts with or without p53 deleted (Col1a2-CreERT:p53$^{fl/fl}$ or Col1a2-CreERT:p53$^{+/+}$) were grown in the presence or absence of FBS (IMDM, 1X Penicillin/Streptomycin, ±10% FBS) for 48 hours at 37^{o}C, 5% CO_2. During pharmacological treatment studies, medium was supplemented with 100μM Pifithrin-α or 0.1μM RITA. After treatment, RNA was isolated and reverse transcription performed with the SV Total RNA Isolation Kit and Reverse Transcription System (Promega), respectively. qPCR was performed using the SensiMix SYBR and Fluorescein Kit (Quantace) and an iQ5 thermal cycler (BioRad). qPCR primer sequences are listed in Supplemental Material

Table 3.1.

Results

To confirm that p53 was upregulated in cardiac fibroblasts at the injury border zone, we performed immunofluorescent staining for p53 in frozen heart sections prepared from Col1a2-CreERT:R26R^{tdTomato} mice 3 days after either ischemia reperfusion or sham injury. We observed that p53 levels were increased 6±0.7 fold (mean±S.E.M, $p<0.005$) in labeled cardiac fibroblasts after IR as compared to sham (Figure 3.3).

To further study the potential role of p53 in MEndoT, we developed an *ex vivo* model system. Others have shown that p53 levels are increased in cells exposed to serum starvation[26,27,28,29] so we sought to determine whether p53 was similarly upregulated in cardiac fibroblasts subjected to serum starvation. To test this, we isolated cardiac fibroblasts and subjected them to serum starvation for 48 hours and compared p53 protein levels between starved and unstarved cells using Western blotting. We found that in the *ex vivo* serum starvation model system p53 levels were increased by 2.3±0.2 fold (mean±S.E.M, $p<0.005$) (Figure 3.4).

After demonstrating that cardiac fibroblasts subjected to serum starvation *ex vivo* upregulated p53 similarly to cardiac fibroblasts following ischemic injury we next determined whether fibroblasts subjected to serum starvation adopted endothelial cell characteristics.

The first assay utilized Matrigel, a well characterized basement membrane substrate that facilitates tube formation of endothelial cells[30]. If cardiac fibroblasts adopt endothelial traits after serum starvation then we hypothesize that they will form endothelial-like structures when cultured on Matrigel in the absence of serum. After sorting labeled cardiac fibroblasts to >95% purity, we seeded the cells on Matrigel and cultured them in the presence or absence of serum for 24 hours. We found that when cultured in medium containing serum, labeled fibroblasts did not form tubes (Figure 3.5a). However, when grown in the absence of serum fibroblasts did form capillary tube-like structures. The endothelial-like structures also expressed the endothelium-specific gene VECAD (Figure 3.5b).

The second functional assay for endothelial cell traits relies on the ability of endothelial cells to internalize acetylated low density lipoprotein (AcLDL). This action is facilitated by the LDL receptor protein expressed and localized to the surface of endothelial

cells. Uptake of AcLDL has been a hallmark of endothelial cells since its discovery almost 30 years ago[31]. Using our serum starvation model system we found that fibroblasts grown in serum replete conditions do not take up AcLDL (Figure 3.5c). However, after 48 hours of serum starvation they do (Figure 3.5d-e). To further demonstrate internalization of AcLDL in MEndoT cells, we utilized confocal imaging in the XZ plane and observed penetration of the fluorophore conjugated AcLDL into the cell (Figure 3.5e).

Together, these finding demonstrate that serum starvation of cardiac fibroblasts recapitulates the increased p53 expression and the MEndoT that takes place after cardiac injury. To further explore whether p53 plays a role in MEndoT in our *ex vivo* model system we utilized gain and loss of function approaches using existing genetic and pharmacological tools.

The loss of function approach was performed using both a pharmacological inhibitor of p53 activity, Pifithrin-α, and a $p53^{fl/fl}$ mouse for genetic deletion. As described previously, Pifithrin-α inhibits p53 activity by blocking its DNA binding region and reducing its function as a transcription factor. To confirm and corroborate our findings with Pifithrin- α we utilized a genetic deletion model in which our tamoxifen inducible fibroblast specific Cre (Col1a2-CreERT) was crossed to $p53^{fl/fl}$ mice. After breeding the mice to attain Col1a2-CreERT:$p53^{fl/fl}$ mice, we were able to use tamoxifen treatment to specifically delete p53 in fibroblasts in a temporally controlled fashion. Using our *ex vivo* serum starvation on Matrigel model, we found that endothelial-like structure formation was significantly reduced in Pifithrin-α treated C57B/6 cardiac fibroblasts and p53 deleted cardiac fibroblasts (Col1a2CreERT:$p53^{fl/fl}$ (CKO)) as compared to PBS treated controls and those with intact p53, respectively (Figure 3.6b-d,f).

For gain of function studies we utilized a small molecule activator of p53 known as RITA. As previously described, RITA has been studied as a method to increase p53 levels in cells by blocking MDM2 mediated poly-ubiquitination and subsequent proteosomal degradation of p53, therefore leading to increased p53 levels. We found that when treated with 0.1μM RITA during serum starvation, the length of endothelial-like tube formation was significantly increased relative to PBS treated controls (Figure 3.6b,e-f).

To confirm our findings from the Matrigel assay, we determined the relative expression of endothelial genes in serum starved cardiac fibroblasts using qRT-PCR (Figure 3.6g). After culturing cardiac fibroblasts isolated from uninjured C57B/6 mice in the presence or absence of serum for 48 hours, we found that endothelial gene expression was increased in serum starved cells. We further assayed whether loss of p53 function, either by culturing the fibroblasts in medium containing 0% serum supplemented with 100μM Pifithrin-α or through genetic deletion of p53 prior to serum starvation, would reduce endothelial gene expression. We observed by qRT-PCR that pharmacological inhibition or genetic deletion of p53 significantly reduced expression of endothelial genes after serum starvation. We also determined whether culturing the cardiac fibroblasts with 0.1μM RITA could enhance endothelial gene expression during serum starvation. We observed that endothelial gene expression was approximately doubled with RITA treatment during serum starvation as compared to PBS treated controls.

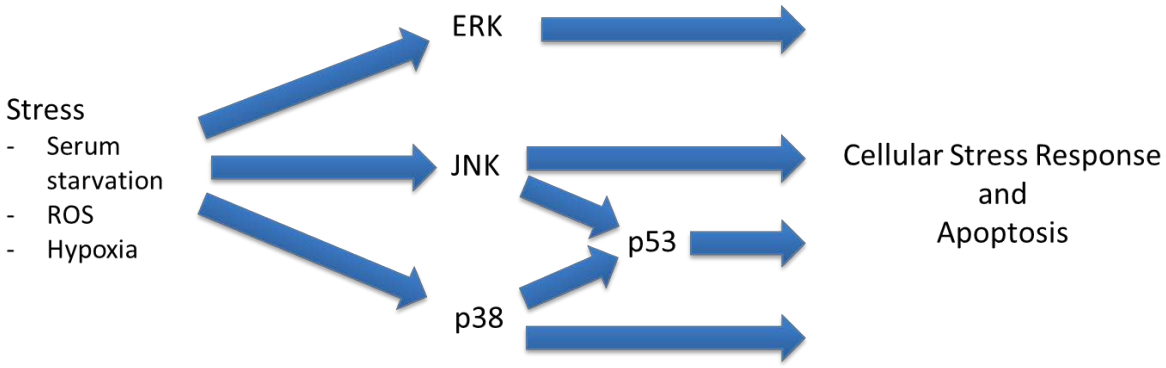
Discussion

Taken together, our findings demonstrate that in an *ex vivo* model of cellular stress, cardiac fibroblasts upregulate p53 expression and adopt an endothelial phenotype. Further,

applying gain and loss of function approaches to our *ex vivo* model system we have demonstrated that MEndoT occurs in a p53 dependent manner.

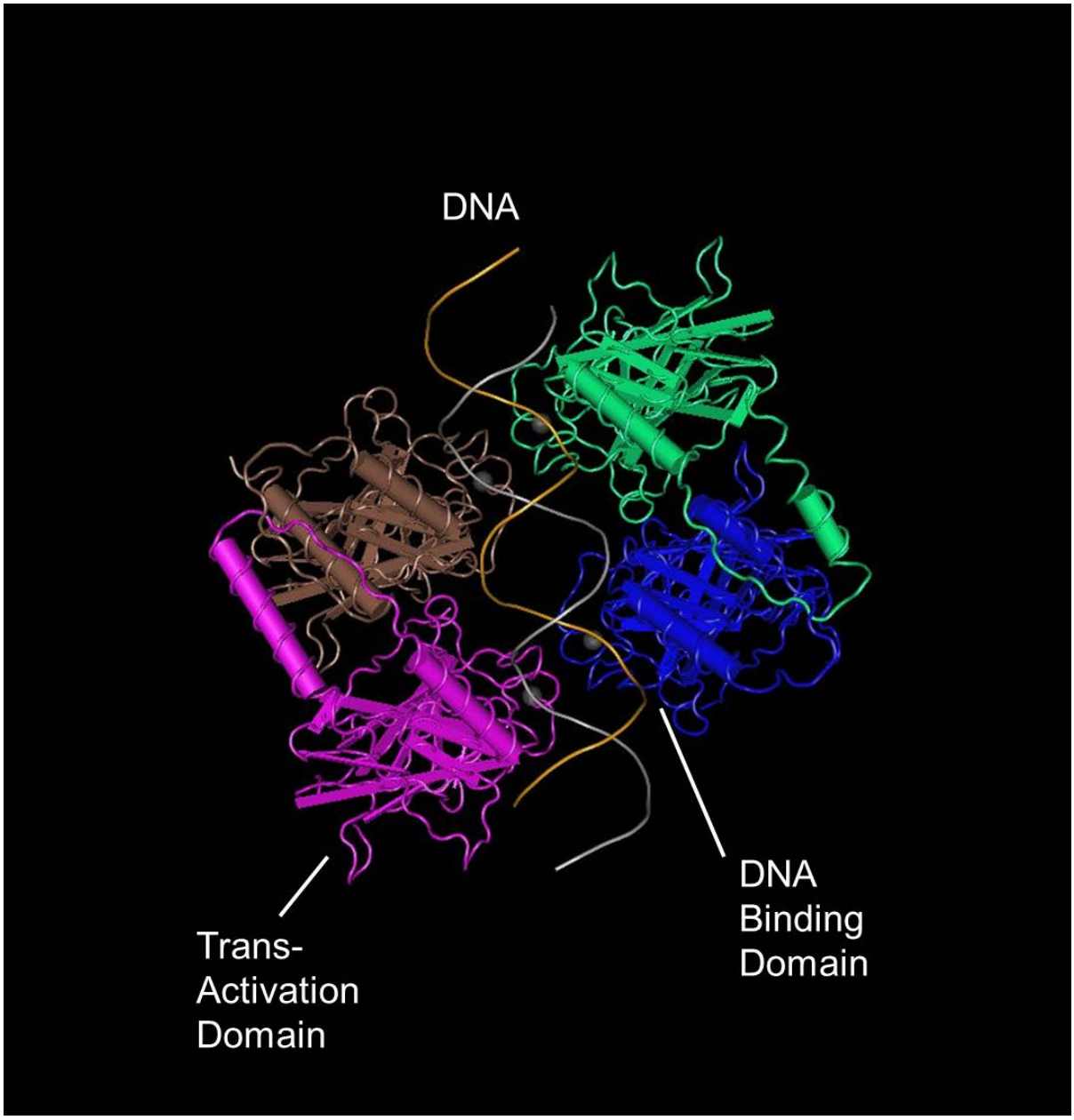
Next we wanted to determine the physiological significance of MEndoT *in vivo*. It is well established that accelerated or augmented neovascularization of the wound region ameliorates post-ischemic injury induced decline in cardiac function. We hypothesized that increasing MEndoT by increasing p53 levels after injury may similarly improve wound healing and cardiac function.

Figure 3.1 MAPK Signaling in Response to Cellular Stress



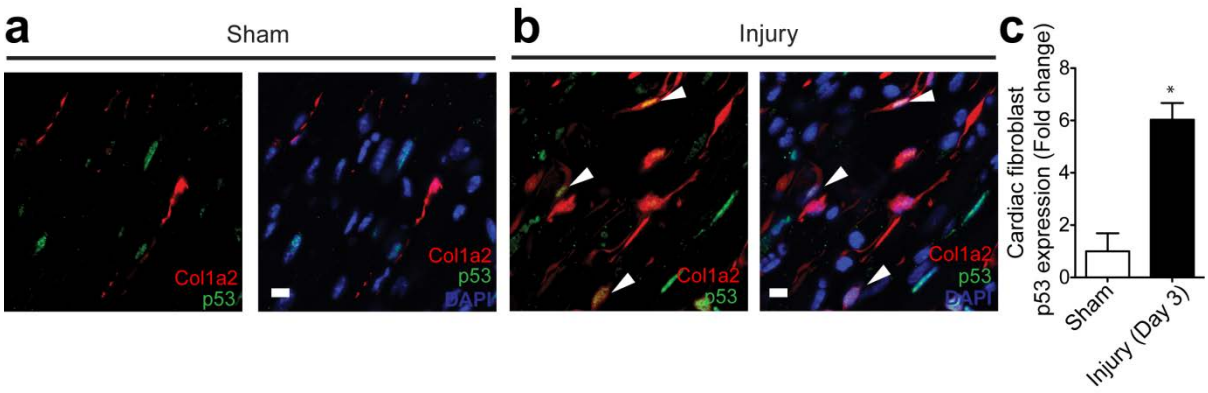
The response to 3 key forms of cellular stress that occur after ischemia reperfusion cardiac injury are mediated by the MAPK signaling pathway.

Figure 3.2 p53 structure



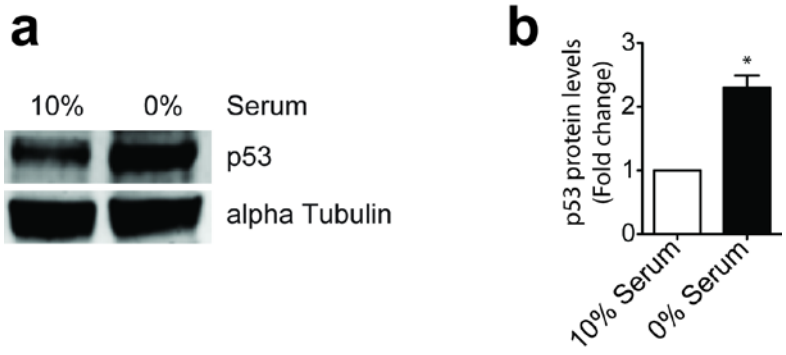
During DNA binding p53 acts as a tetramer (subunits shown in brown, green, pink and blue). The locations of the trans-activation domain (TAD) and DNA binding domain are indicated by white lines. DNA is shown as an orange and gray helix.

Figure 3.3 Cardiac fibroblasts upregulate p53 after cardiac injury.



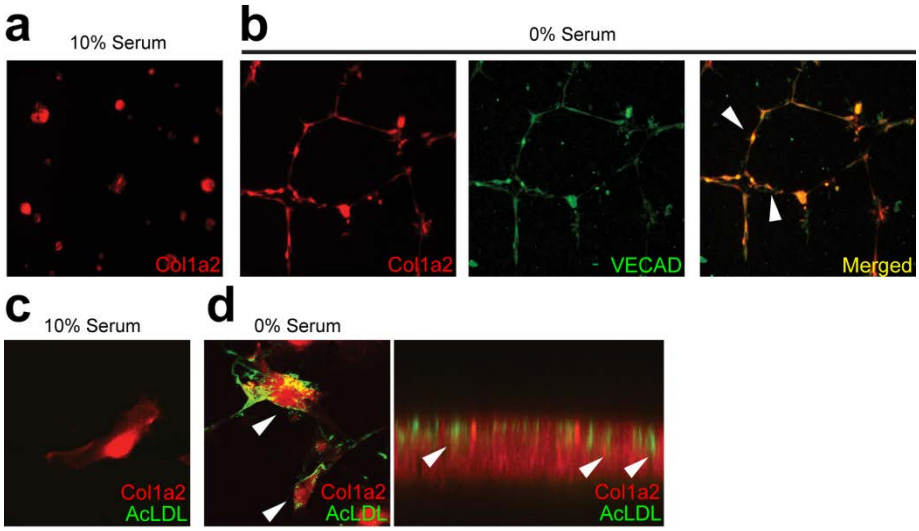
(a-b) Col1a2-CreERT:R26R^{tdTomato} mice underwent either **(a)** sham or **(b)** ischemia reperfusion injury. Hearts were harvested 3 days after injury and immunostained for p53 (Arrowheads indicate labeled fibroblasts expressing p53, n=3, scale bar = 10μm). **(c)** Fold change of p53 expression in labeled cardiac fibroblasts at the injury border zone (n=3, *p<0.005).

Figure 3.4 Serum starvation leads to upregulation of p53 in cardiac fibroblasts.



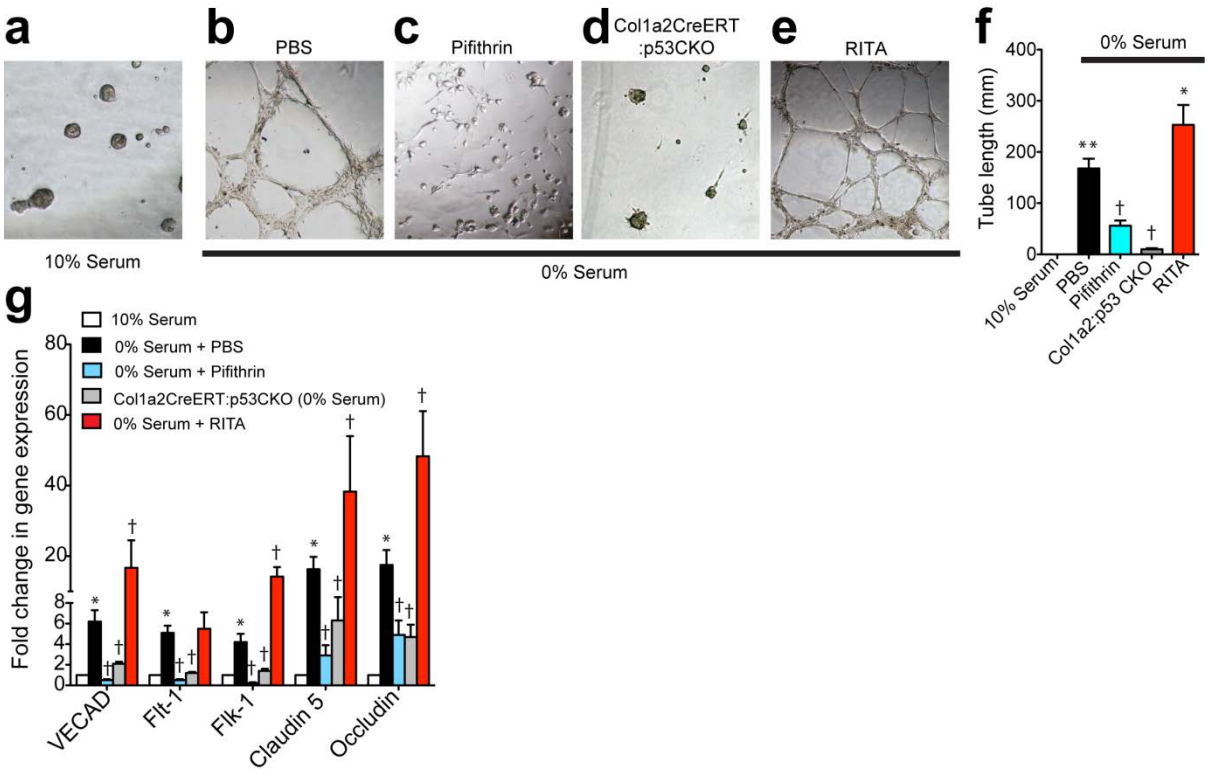
(a-b) Cardiac fibroblasts isolated from uninjured C57B/6 mice were grown in the presence or absence of serum for 48 hours, protein was isolated, and Western blot analysis was performed. **(a)** Western blot demonstrating p53 levels in cells cultured in 10% or 0% serum with alpha Tubulin as a loading control. **(b)** Densitometry analysis quantitating the fold change increase of p53 in fibroblasts cultured in 0% serum as opposed to 10% serum (n=4, *$p<0.005$).

Figure 3.5 Cardiac fibroblasts adopt an endothelial phenotype in response to serum starvation.



(a-b) Cardiac fibroblasts isolated from uninjured Col1a2-CreERT:R26R^{tdTomato} mice were cultured on Matrigel for 24 hours in the **(a)** presence or **(b)** absence of serum (arrowheads indicate colocalization of tdTomato label and immunostained VECAD in endothelium-like structures, n=4). **(c-d)** Col1a2-CreERT:R26R^{tdTomato} cardiac fibroblasts were cultured in the **(c)** presence or **(d)** absence of serum for 48 hours and imaged 4 hours after incubation with Alexa488 conjugated AcLDL. Right panel of **(d)** is in the XZ plane to shown internalization (arrowheads indicate colocalization of fluorophores, n=4).

Figure 3.6 p53 mediates MEndoT in an *ex vivo* model system of cellular stress.



(a-f) Endothelium-like structure formation of cardiac fibroblasts cultured on Matrigel for 24 hours in medium containing **(a)** 10% serum, **(b-e)** 0% serum. Fibroblasts were cultured with **(c)** 100μM Pifithrin, **(e)** 0.1μM RITA, or **(d)** after genetic deletion of p53 (n=3). **(f)** Total length of endothelium-like structures from **(a-e)** quantitated using ImageJ software (**p<0.005 compared to cells cultured in 10% serum, †p<0.005 compared to cells cultured in 0% serum plus PBS, *p<0.05 compared to cells cultured in 0% serum). **(g)** qPCR for expression of endothelial genes in cardiac fibroblasts treated as described (*p<0.005 compared to cells cultured in 10% serum, †p<0.05 compared to cells cultured in 0% serum plus PBS, n=8).

Supplemental Material

Table 3.1 qPCR Primer Information

		Primer Sequence (5'-3')	
Gene Name	**mRNA Accession Number**	**Forward**	**Reverse**
VECAD	NM_009868.4	tgcccaccatcgccaaaaga	tttcccacatagtggggcag
Flt-1	NM_010228.3	ggtgtctgcttctcacagga	agagtctggcctgcttgcat
Flk-1	NM_010612.2	tcagctatgccggcatggtc	gcgggggctcagaatcaca
Claudin 5	NM_013805.4	gccgtcgggtgagcattcag	gccaggatcaagcccaccca
Occludin	NM_008756	gcctaaactacccttatagt	gctcttgggtctgtatatcc

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CHAPTER 4 MEndoT plays a functional role in cardiac repair

Overview

Objective: The goal of this study is to determine the physiological significance of MEndoT after acute ischemic cardiac injury. After demonstrating that p53 regulates MEndoT *ex vivo*, we use gain and loss of function approaches *in vivo* to determine what effects MEndoT has on wound healing and cardiac function after injury.

Methods and Results: We utilized p53 based gain and loss of function approaches to determine the physiological role of MEndoT after cardiac injury. By genetic deletion of p53 in fibroblasts prior to injury, we observed a reduction in MEndoT and a decrease in vascularity at the injury border zone. We also observed a significant decrease in cardiac function and increased scarring in mice with p53 deleted prior to injury as compared to mice with intact p53. Conversely, when treated with a p53 activator, RITA, after cardiac injury mice showed increased MEndoT and increased vascularity at the site of injury when compared to PBS treated control animals. RITA treated injured animals also showed improved cardiac function and reduced scar formation as compared to control animals.

Conclusions: Here we show that MEndoT plays a significant role in the wound healing process after acute cardiac injury. Inhibition of MEndoT after cardiac injury led to significant declines in vascularity and heart function and increased scar formation. Our observations

suggest a novel therapeutic strategy for enhancing cardiac repair after acute ischemic injury that can be used to ameliorate decline in post-injury cardiac function and reduce scarring.

Introduction

Myocardial infarction is the leading cause of morbidity and mortality in the United States[1]. Revascularization at the site of ischemic cardiac injury is essential to normal wound healing. The return of regulated blood flow delivers necessary oxygen and nutrients to surviving cells bordering the site of injury and fosters the proliferation of fibroblasts and endothelial cells allowing reparative fibrosis. Accelerated neovascularization of the injury region promotes rapid recovery of the ischemic heart. Current research efforts have focused on the use of cellular reprogramming[2,3], targeting endothelial cell proliferation to improve angiogenesis[4], localization of circulating endothelial progenitor cells[5,6,7,8] and resident cardiac stem cells[9,10] to increase the pool of endothelial cells in the injured heart, thereby increasing vascularity at the site of injury. Evaluation of each of these strategies is still underway but to date each method has demonstrated improvements in cardiac function after injury.

Given our findings that cardiac fibroblasts can adopt an endothelial fate after injury and that the process is mediated by p53 *ex vivo*, we sought to determine what role MEndoT plays in the physiology of wound healing. Since MEndoT contributes to vascularity after injury, we also sought to determine whether improving MEndoT would lead to increased neovascularization at the site of injury and improve cardiac function.

To determine how manipulations of MEndoT would affect vascularity and cardiac function we used gain and loss of function approaches. By deleting p53 prior to injury in cardiac fibroblasts we sought to determine what effect loss of MEndoT would have on

vascularity at the site of injury and associated cardiac function. Conversely, using RITA to increase p53 levels after injury we sought to determine if increasing MEndoT could improve vascularity and provide a therapeutic benefit.

Materials and Methods

Generation of transgenic mice

All animal experiments were conducted in accordance with the University of North Carolina Institutional Animal Care and Use Committee (IACUC) guidelines. Collagen1a2-CreERT:R26R^{tdTomato} mice were obtained by breeding Collagen1a2-CreERT mice with R26R^{tdTomato} mice. Collagen1a2-CreERT:R26R^{tdTomato}:p53CKO mice were obtained by crossing and backcrossing Collagen1a2-CreERT:R26R^{tdTomato} mice with p53$^{fl/fl}$ mice[11]. Genetic labeling or genetic labeling and deletion of p53 was achieved by intraperitoneal injection of 8-10 week old Collagen1a2-CreERT:R26R^{tdTomato} or Collagen1a2-CreERT:R26R^{tdTomato}:p53CKO mice, respectively, with1mg tamoxifen (Sigma) intraperitoneally daily for 10 days. 5 days after cessation of tamoxifen the mice either underwent ischemia reperfusion or sham injury.

Cardiac injury model

Sham or ischemia reperfusion injury was performed as previously described on page 18.

RITA and PBS treatment after injury

Injured animals were treated with 0.3mg/kg RITA or PBS once daily for 3 to 7 days beginning 24 hours after injury with the number of days of injection depending on the time-point for heart harvest.

Immunofluorescent staining , confocal imaging, and quantitation

Frozen sections (7µm thick) were prepared from hearts isolated 3, 7, or 14 days after injury from ischemia reperfusion or sham injured mice. Sections were stained, imaged and

quantitated as described on page 19 with the exception that the primary antibodies used were VECAD and p53 (Abcam). The total number of VECAD positive cells per microscopic field were counted using ImageJ software (NIH).

Echocardiography

Mice were preconditioned to conscious restraint 5 minutes daily for the 3 days preceding the echocardiographic procedure. Prior to echocardiography, the hair over the anterior chest was removed using depilation cream, warmed, and Aquasonic gel applied. Conscious mice were held firmly by hand for the duration of the procedure (5-10 minutes). The probe was positioned over the chest in a parasternal position. Parasternal long axis B- and M-mode images were recorded. Analysis of echocardiograms was performed using Vevo software (FujiFilm).

Masson's Trichrome staining and fibrotic area measurement

Masson's Trichrome staining was performed as described[12] on cardiac sections prepared 7 or 14 days after injury. The measurement of fibrotic area was performed using ImageJ software (NIH). The percentage of fibrotic area within the left ventricle was calculated by dividing the fibrotic area within the left ventricle by the total area of the left ventricle.

Results

To determine whether p53 regulates MEndoT *in vivo*, we crossed and back-crossed Col1a2-CreERT:R26R^{tdTomato} mice with mice that have both p53 alleles floxed. Progeny mice, Col1a2-CreERT:R26R^{tdTomato}:p53$^{fl/fl}$, at 8-10 weeks of age were given tamoxifen daily for 10 days to generate mice with fibroblasts deficient in p53, henceforth referred to as Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice. The presence of the tdTomato reporter enables us to label fibroblasts that are deficient in p53. At 8-10 weeks of age, these mice received

intraperitoneal injection of tamoxifen for 10 days and 5 days after the cessation of tamoxifen they underwent ischemia reperfusion or sham injury. 3 days after injury the hearts were harvested, sectioned and immunostained for p53 as were their counterparts with intact p53 (Figure 4.1a-b). We observed that after injury, Col1a2-CreERT:R26R^{tdTomato} mice with intact p53 had a 6±0.64 fold increase (mean±S.E.M.) in p53 expression at the injury border zone as compared to mice undergoing sham injury. However, in mice with p53 deleted prior to injury p53 expression did not increase significantly as compared to sham injured animals with intact p53 (1.25±0.42 fold (mean±S.E.M.), p=0.23) (Figure 4.1c). This confirmed that p53 was effectively deleted in our p53 CKO model system.

To further understand whether p53 deletion would inhibit MEndoT, as we observed in our *ex vivo* model system, we immunostained cardiac sections prepared 3 days after injury and found that the percentage of labeled cardiac fibroblasts in Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice that underwent MEndoT at the injury border zone was decreased by 57% as compared to Col1a2-CreERT:R26R^{tdTomato} mice with intact p53 (Figure 4.2a-c). We also observed that the total number of VECAD positive endothelial cells at the injury border zone was reduced by approximately 41%.

Since increased vascular density after cardiac injury is associated with improved heart function and reduced scarring we next sought to determine whether the decrease in vascularity associated with reduced MEndoT had a negative effect on cardiac function after injury. We performed echocardiographic analysis prior to and 7 days after cardiac injury in Col1a2-CreERT:R26R^{tdTomato}:p53CKO and Col1a2-CreERT:R26R^{tdTomato} mice with intact p53. We found that fractional shortening and ejection fraction was significantly reduced 7 days after injury in Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice as compared to Col1a2-CreERT:R26R^{tdTomato} mice with intact p53 (Figure 4.3a-b). We also compared the extent of cardiac scarring 14 days after injury by staining cardiac sections with Masson's Trichrome.

We found that the fibrotic area as a percentage of left ventricular area was significantly increased in Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice compared to controls (Figure 4.3c-d).

Since the loss of p53 led to a reduction in MEndoT, decrease in vascularity at the border zone, decline in heart function and increased scarring, we next sought to determine whether increasing p53 levels after injury would have the opposite effect. To do so, we injected Col1a2-CreERT:R26R^{tdTomato} mice with either 0.3mg/kg RITA, a p53 activator, or PBS beginning 24 hours after injury. The hearts were harvested 3 days after injury, sectioned and immunostained for p53. We observed that p53 expression in labeled cardiac fibroblasts was increased by 13±0.8 fold in RITA treated ischemia reperfusion injured animals as compared to sham injured animals. This was more than double the p53 expression in cardiac fibroblasts of PBS treated Col1a2-CreERT:R26R^{tdTomato} mice, which was approximately 6 fold more than sham injury (Figure 4.4).

To determine the effect of increased p53 expression at the injury border zone on MEndoT, we immunostained cardiac sections isolated 3 days after injury in RITA and PBS treated Col1a2-CreERT:R26R^{tdTomato} mice for VECAD. We found that the percentage of cardiac fibroblasts expressing the endothelial marker were increased to 51±4% in RITA treated animals as compared to 35±3% in PBS treated animals (mean±S.E.M.). We also found that the total number of VECAD positive endothelial cells at the injury border zone was significantly increased (Figure 4.5).

To understand whether increased MEndoT and vascularity had an effect on scarring and heart function, we utilized Masson's Trichrome staining and echocardiography respectively. We compared fibrosis in sections prepared from hearts isolated 7 days after injury in RITA and PBS treated animals. We found that the percentage of left ventricular

scarring was reduced from 39±4% in PBS treated animals to 10±5% in RITA treated animals (mean±S.E.M.). This represents an approximately 75% decrease in the amount of fibrosis (Figure 4.6a-b). We compared echocardiographic measurements of fractional shortening and ejection fraction prior to and 7 days after injury and found that in RITA treated animals both measures were significantly improved as compared to PBS treatment (Figure 4.6c-d).

To demonstrate that the increased MEndoT observed after administration of RITA was dependent upon p53 in cardiac fibroblasts, we injected RITA into tamoxifen treated Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice after cardiac injury and observed that the ability of RITA to enhance MEndoT was significantly attenuated. The amount of MEndoT observed was not statistically different than that observed in tamoxifen treated Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice not treated with RITA (Figure 4.7), demonstrating that RITA enhanced MEndoT primarily occurs through the activation of p53 in cardiac fibroblasts.

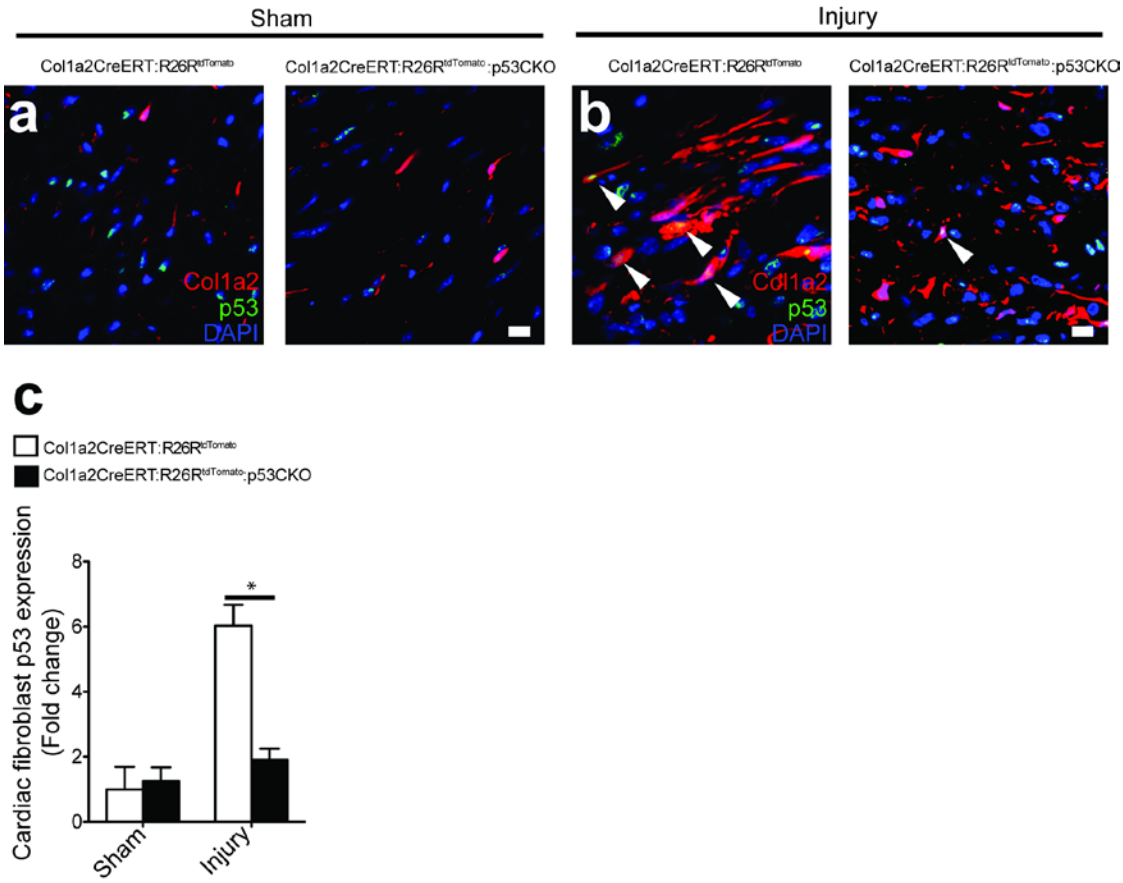
Discussion

Using both gain and loss of function approaches we demonstrated *in vivo* that MEndoT is a p53 mediated process after acute cardiac injury. We further demonstrated that MEndoT plays a functional role in the physiology of wound healing. By reducing MEndoT by genetically deleting p53 in fibroblasts prior to injury, we observed reduced post infarct vascularity at the border zone, increased scarring and a decline in heart function. However, when we increased MEndoT using the p53 activator RITA, we found just the opposite effect. Taken together, our findings indicate that MEndoT takes place after cardiac injury and is mediated by p53. Further, we have discovered that modulation of p53 levels after cardiac injury and subsequent increases in MEndoT can significantly enhance cardiac function. Given the previously established link between improved post-infarct angiogenesis and

improvements in heart function and scarring, it is not surprising that increases in vascularity attributed to MEndoT could enhance cardiac function and reduce scarring after injury.

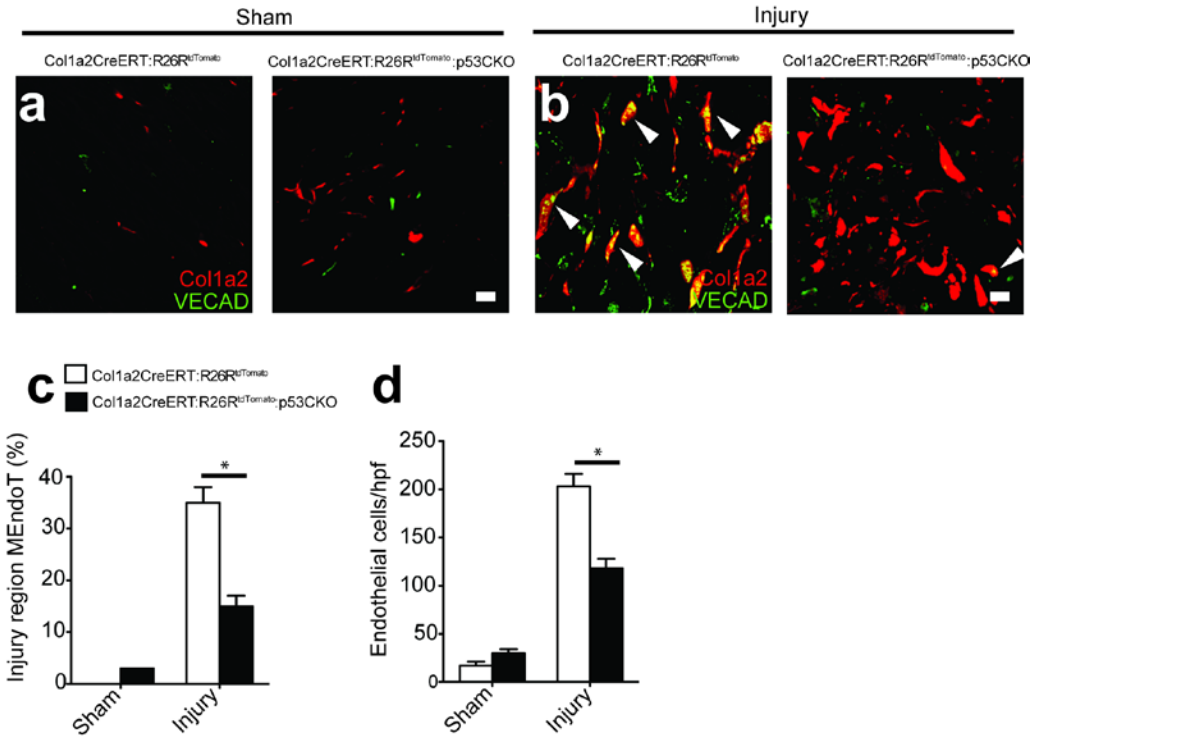
While our observations do not exclude the possibility that perturbations of other aspects of p53 mediated biology may lead to adverse effects on post-injury wound repair and cardiac function, it is also not surprising that given the critical role of neovascularization after ischemic injury that a disruption of MEndoT would lead to decreased vascular density and diminished heart function.

Figure 4.1 Genetic deletion of p53 reduces p53 expression in cardiac fibroblasts.



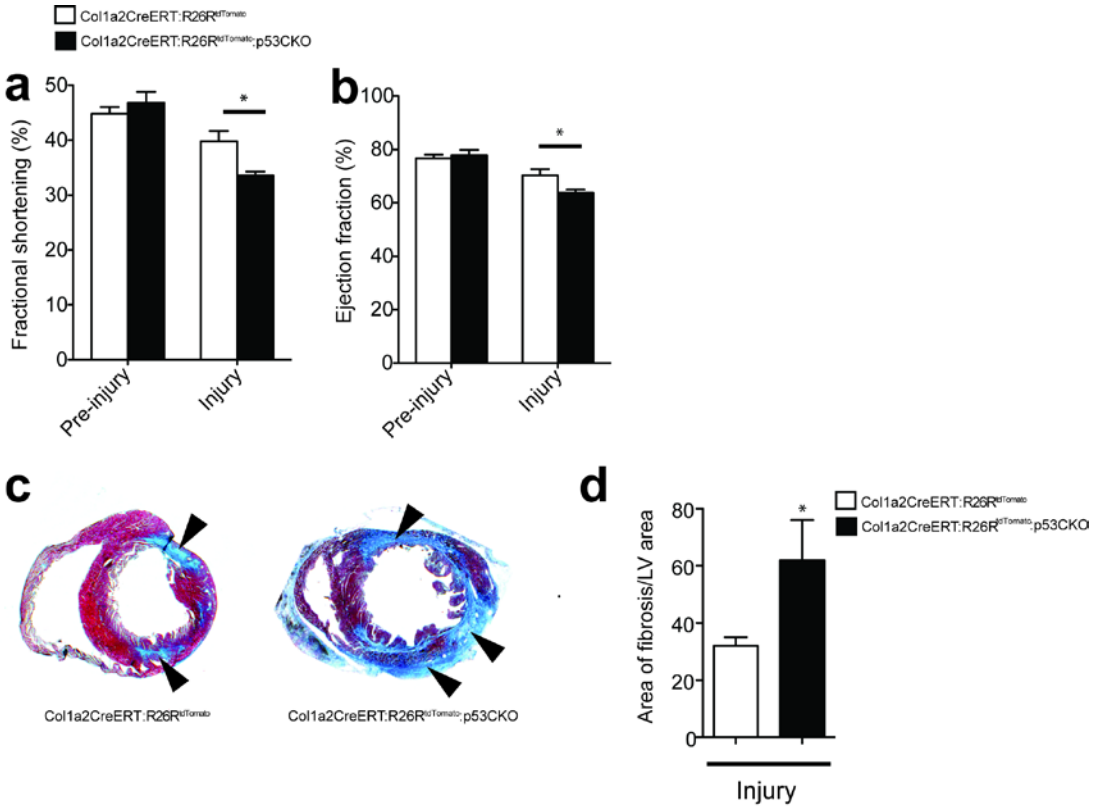
(a-b) Immunostaining of p53 in cardiac sections prepared from **(a)** sham and **(b)** ischemia reperfusion injured Col1a2-CreERT:R26R^{tdTomato} or Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice 3 days after injury (arrowheads show expression of p53 in labeled cardiac fibroblasts, n=4, scale bar = 10μm) **(c)** Quantitation of p53 expression levels relative to sham injured Col1a2-CreERT:R26R^{tdTomato} mice ($p<0.005$, n=4).

Figure 4.2 Genetic deletion of p53 in cardiac fibroblasts leads to a reduction in MEndoT and fewer endothelial cells at the border zone of injury.



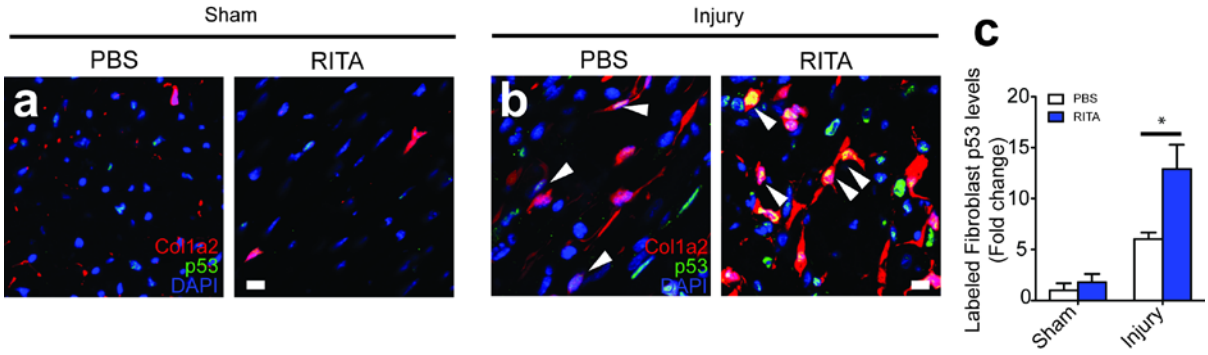
(a-b) Immunostaining of VECAD in cardiac sections prepared from **(a)** sham and **(b)** ischemia reperfusion injured Col1a2-CreERT:R26R^{tdTomato} or Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice (arrowheads show expression of VECAD in labeled cardiac fibroblasts, n=4, scale bar = 10μm) **(c)** Quantitation of the percentage of labeled cardiac fibroblasts expressing VECAD (*p<0.005, n=4). **(d)** Comparison of the total number of VECAD expressing cells per high power microscopic field in sham and ischemia reperfusion injured Col1a2-CreERT:R26R^{tdTomato} or Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice (* p<0.005, n=5).

Figure 4.3 Inhibition of MEndoT leads to reduced heart function and increased scarring after acute cardiac injury.



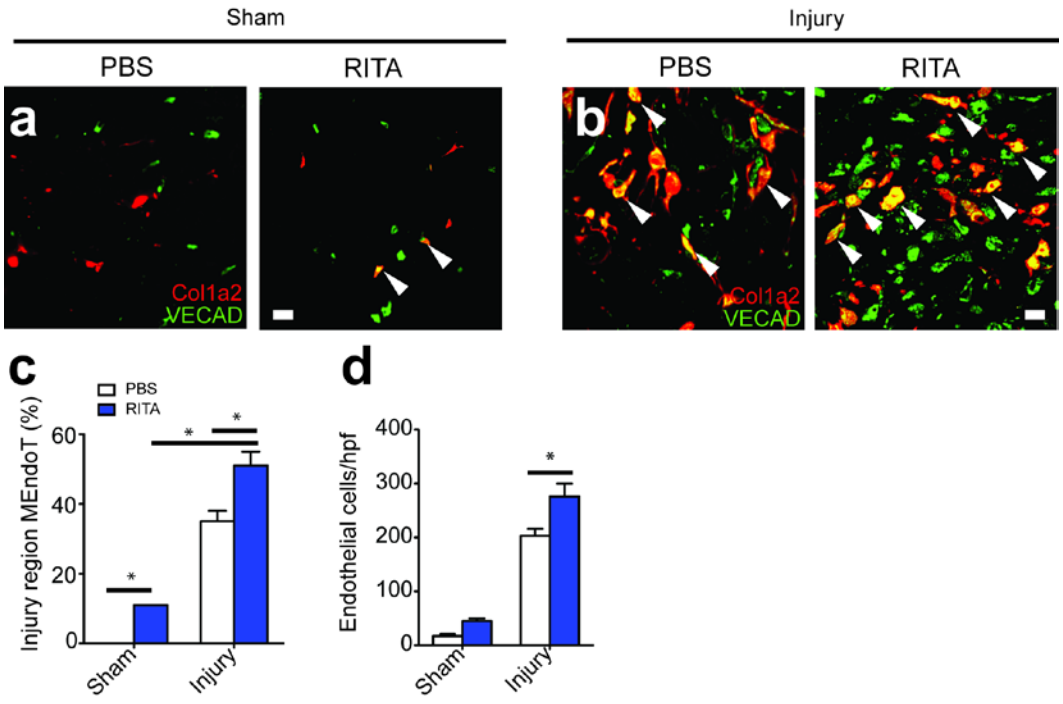
(a-b) Echocardiographic measurement of **(a)** fractional shortening and **(b)** ejection fraction prior to and 7 days after ischemia reperfusion injury in Col1a2-CreERT:R26R^{tdTomato} or Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice (* $p<0.05$, n=9 for Col1a2-CreERT:R26R^{tdTomato} mice and n=8 for Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice). **(c)** Masson's Trichrome staining of heart sections prepared from Col1a2-CreERT:R26R^{tdTomato} or Col1a2-CreERT:R26R^{tdTomato}:p53CKO mice 14 days after ischemia reperfusion injury (arrowheads indicate fibrotic area (blue), n=4). **(d)** Quantitation of the fibrotic area as a percentage of the left ventricular area (* $p<0.05$, n=4).

Figure 4.4 RITA treatment after acute cardiac injury leads to increased p53 expression in cardiac fibroblasts.



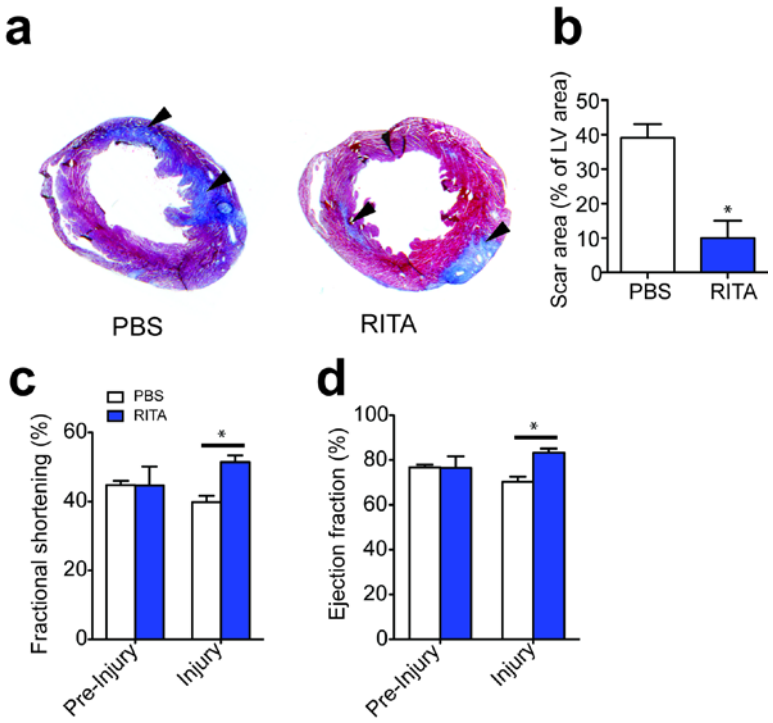
(a-b) Immunostaining of p53 in cardiac sections prepared 3 days after **(a)** sham or **(b)** ischemia reperfusion injury in PBS or RITA treated Col1a2-CreERT:R26R^{tdTomato} mice (arrowheads show expression of p53 in labeled cardiac fibroblasts, n=4, scale bar = 10μm) **(c)** Quantitation of p53 expression levels relative to PBS treated sham injured Col1a2-CreERT:R26R^{tdTomato} mice ($p<0.005$, n=4).

Figure 4.5 RITA treatment after cardiac injury increases MEndoT and vascularity at the site of injury.



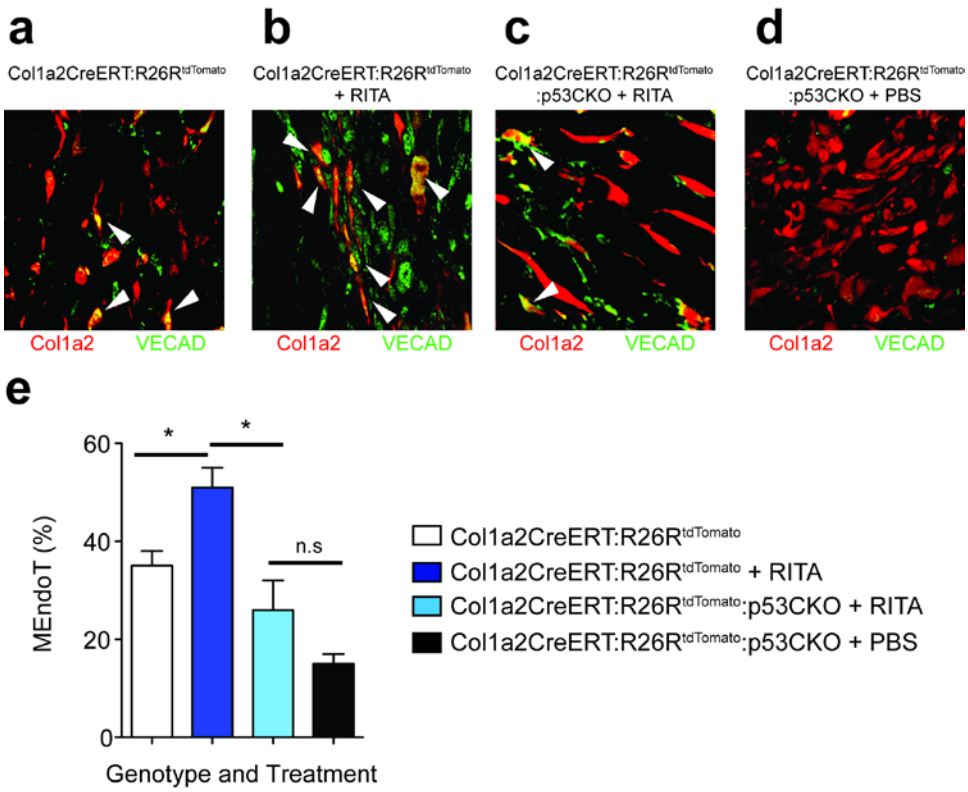
(a-b) Immunostaining of VECAD in cardiac sections prepared 3 days after **(a)** sham and **(b)** ischemia reperfusion injury in PBS or RITA treated Col1a2-CreERT:R26R^{tdTomato} mice (arrowheads show expression of VECAD in labeled cardiac fibroblasts, n=4, scale bar = 10μm) **(c)** Quantitation of the percentage of labeled cardiac fibroblasts expressing VECAD (*p<0.05, n=4). **(d)** Comparison of the total number of VECAD expressing cells per high power microscopic field in sham and ischemia reperfusion injured PBS or RITA treated Col1a2-CreERT:R26R^{tdTomato} mice (* p<0.05, n=4).

Figure 4.6 Improved vascularity after RITA treatment is associated with reduced scar formation and improved cardiac function.



(a) Masson's Trichrome staining of heart sections prepared from PBS and RITA treated Col1a2-CreERT:R26R^{tdTomato} mice 7 days after ischemia reperfusion injury (arrowheads indicate fibrotic area (blue), n=4). **(b)** Quantitation of the fibrotic area as a percentage of the left ventricular area (* $p<0.05$, n=4). **(c-d)** Echocardiographic assessment of **(c)** fractional shortening and **(d)** ejection fraction prior to and 7 days after ischemia reperfusion injury in PBS or RITA treated Col1a2-CreERT:R26R^{tdTomato} mice (* $p<0.05$, n=8).

Figure 4.7 RITA treatment in p53CKO mice does not significantly improve MEndoT.



(a-d) Immunostaining for VECAD in heart sections prepared 3 days after ischemia reperfusion injury in **(a)** Col1a2-CreERT:R26R^{tdTomato}, **(b)** Col1a2-CreERT:R26R^{tdTomato} + RITA, **(c)** Col1a2-CreERT:R26R^{tdTomato}:p53CKO + RITA or **(d)** Col1a2-CreERT:R26R^{tdTomato}:p53CKO + PBS mice. **(e)** Quantitation of MEndoT percentage by genotype and treatment listed (* $p<0.05$, n.s. = not significant, n=4 per group).

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CHAPTER 5 Conclusions and Perspectives

Summary of Findings

In this dissertation I have described a novel phenomenon where fibroblasts undergo Mesenchymal-to-Endothelial Transition (MEndoT) in response to acute ischemic injury and contribute to vasculogenesis and the revascularization of the injured myocardium. In Chapter 2 I described the use of fate mapping techniques in the determination that cardiac fibroblasts at the injury border zone express endothelium-specific markers and integrate into the vasculature. To date, this phenomenon has not been described either during development or after injury in any organ system. In Chapter 3 I describe an *ex vivo* model system of cellular stress that is able to recapitulate the MEndoT seen *in vivo*. Further, I describe how p53, a key cell cycle regulator, regulates MEndoT. This finding expands our burgeoning understanding of how p53 mediates cellular phenotype conversion. In Chapter 4 I demonstrate the physiological significance of MEndoT in wound healing. By manipulating p53 and thereby MEndoT, I show that increasing MEndoT leads to increased vascularity at the site of injury and improved cardiac function with reduced scarring.

Limitations

While these observations are novel and stress the potential therapeutic implications of MEndoT they also come with a number of caveats, some of which will hereafter be described.

Murine model

As previously described, the research presented utilized a Collagen1a2-CreERT based labeling system. While we validated the specificity and labeling efficiency of the model, there are some potential challenges which could affect the utility of our findings. First, as previously described, there are no known markers that are truly specific to fibroblasts. While we sought to validate the system using Thy1 expression characteristic of fibroblasts, there are several other fibroblast markers that could have been used to confirm that the labeled cells are fibroblasts. This would have been useful since Collagen1a2 has been demonstrated to be expressed in some other cells[1]. Confirmation of the fibroblast expression profile could have been achieved by assessing the expression of other targets in the labeled cells such as Fsp1, DDR2, or Vimentin using flow cytometry, immunostaining, or both. Secondly, the observations could have been corroborated using another fibroblast marker Cre. As an example, in early work we utilized a non-inducible Fsp1-Cre in *in vivo* and *ex vivo* model systems to confirm the results, though those results were not presented.

Another potential limitation to the existing Col1a2-CreERT model was the efficiency of the Cre recombinase both during labeling and p53 gene deletion. After tamoxifen injection, we observed that approximately 70% of isolated fibroblasts were labeled. With another Cre line or potentially with an altered tamoxifen administration regime in the Col1a2-CreERT model, it is possible that the labeling efficiency could have been improved which may have provided a more accurate measurement of the percentage of cells undergoing MEndoT. Further, after p53 deletion we still observed p53 expression in labeled fibroblasts though expression was reduced by approximately 50%. With an improved model system, it may have been possible to more fully ablate p53 and its expression to obtain a closer approximation of how the presumably total loss of MEndoT would affect wound healing. While we could have used a global p53 knock-out mouse model to achieve similar results,

we chose to use the less efficient conditional knock-out system because it was less likely to alter the mouse physiology (i.e. with spontaneous tumor formation) and provide a closer view of how MEndoT affects wound healing under normal physiological conditions.

Another more general limitation of working in a murine model is the caveat that observations made in one mouse strain may not be reproducible across strains or, more importantly, across species to humans. One of the ultimate goals of this research is to identify therapeutics for human patients. Given the prevalence of myocardial infarction and the burden of the disease on the human population and the healthcare system, we would like to find a treatment to improve the outcome of human patients, not mice.

Unexpected and off-target effects of p53 manipulation

As previously stated, p53 is part of the complex and highly regulated MAPK pathway. Another limitation of the work described is that perturbations in p53 biology can have unexpected and off-target effects, either within the affected cells or in neighboring cells.

As a specific example, in the simplified context of our *ex vivo* work, we showed that manipulation of p53 had very direct effects on the adoption of endothelial traits during MEndoT. However, in the body cells operate in a complex milieu replete with various extracellular signals. It is likely that the direct effects we demonstrated in the *ex vivo* model may be mitigated (or enhanced) by other factors at the site of injury. The increased complexity of the *in vivo* model may have a direct effect on the presented results. As an example, while deletion of p53 prior to injury did not have an apparent effect on heart function, after injury, heart function was decreased. While we attribute that result in the context of other findings on the effects of fibroblasts on vascularity in wound healing[2,3,4], it is possible that p53 deletion in fibroblasts could have an unexpected and previously unknown

role in maintaining heart function after injury. Similarly and perhaps more directly, deletion of p53, a key cell cycle checkpoint, could have led to the increased proliferation of fibroblasts, which in turn led to the increased scarring observed as well as potentially adverse consequences for heart function.

While we used gain and loss of function approaches to strengthen our claims that changes in post-injury vascularity associated with MEndoT can lead to reduced scarring and improved cardiac function after injury, it is also possible that RITA treatment after injury functionally reduced the proliferation of fibroblasts and the reduced scarring was a consequence. That reduction in scarring may also have led to improved heart function through an unknown mechanism.

Alternatively, RITA treatment could have had off-target effects that led to improvements in heart function. As an example, the RITA administered systemically 1-3 days after injury could locally have affected the biology of a number of different cell types (i.e. neutrophils, macrophages, endothelial cells, cardiomyocytes) at the site of injury or even other organs such as the kidneys that regulate blood pressure. We cannot exclude that off target effects may have played a role in the improvements in heart function observed after RITA treatment. Until the technology used to activate p53 can be restricted to fibroblasts, it is difficult to perfectly attribute all of the changes in heart function and wound healing observed solely to MEndoT.

Analysis of cells in aggregate

One of the other limitations of this work is that much of the analysis was performed on an aggregate pool of cells. While we observed that approximately 30% of labeled fibroblasts at the border zone were able to adopt an endothelial phenotype after injury, it

begs the question of why the other 70% of labeled fibroblasts, ostensibly exposed to the same cellular context, did not. The *ex vivo* work that determined the mechanism governing MEndoT was described using techniques that treat cells in a pool. However, to better understand the regulation of MEndoT, a single cell based approach may offer additional benefits. Current limitations on the use of high-throughput single cell analysis effectively reduce the ability to study MEndoT on large numbers of individual cells. In the future, as better and more affordable analysis techniques become available, it may be interesting to study and characterize cell subpopulations and understand why, or why not, they undergo MEndoT.

Future Directions

The discovery of MEndoT and the elucidation of its mechanical underpinnings presented in this dissertation are by no means an exhaustive study of all aspects of this form of cellular transition. There are several key questions yet to be addressed in order to more fully understand the process of MEndoT and its implications. In this section, a series questions addressing other associated areas of scientific exploration are proposed.

Does MEndoT occur in other organ systems?

During our research we demonstrate that MEndoT occurs after ischemic cardiac injury and contributes to neovascularization at the injury border zone. One question we did not address was whether MEndoT occurs in the wound healing process in other organ systems. As an example, during granulation tissue formation in the wound healing response of the skin, local fibroblasts and endothelial cells have been shown to communicate to coordinate scar formation in a fashion similar to what takes place in the heart. It may be

possible that when exposed to alternate forms of cellular stress local fibroblasts in other parts of the body may adopt an endothelial phenotype. In the case of skin injury, exposure of fibroblasts to the outside environment may lead to MEndoT as p53 becomes upregulated. Using the same murine model, injury to other organ systems could be studied to determine whether MEndoT occurs in other parts of the body.

The idea of MEndoT in response to injury could also be extended to cancer. During cancer, tumor growth often leads to local tissue hypoxia[5,6]. Given the pervasive nature of fibroblasts in the body, it is also possible that local fibroblasts, when exposed to hypoxic conditions similar to those after ischemic cardiac injury, could adopt an endothelial phenotype and contribute to the vasculature supplying the growing tumor. This may even provide an alternative explanation as to why anti-angiogenic therapies have limited success in the treatment of cancers[7,8,9,10].

The concept of MEndoT also need not be limited to injury. While it has not been formally described, it is possible that MEndoT may also occur during development. Unlike MET, which has been shown to take place during kidney formation[11,12], MEndoT has not been expressly shown. After demonstrating that MEndoT can take place after injury, it may further our understanding of biology to determine whether it also takes place in during development.

Can fibroblasts adopt other fates?

We show that cardiac fibroblasts can adopt an endothelial fate after ischemic injury. That leads to the question of whether fibroblasts can adopt other fates as well. While not covered in the context of this work, we can address that question in a limited way based on other studies we have conducted. In my earlier work, I observed that cardiac fibroblasts can

also adopt an epithelial fate in response to ischemic cardiac injury. This study was proposed to, and funded by, the American Heart Association in my pre-doctoral fellowship (*Role of Mesenchymal-to-Epithelial Transition in Cardiac Repair* - Award #11PRE7720016). Preliminary data even suggest that the MET that takes place in the heart is mediated by a similar stress response mechanism. Whether the subset of fibroblasts undergoing MET is the same as the subset undergoing MEndoT has not been determined. However, it is entirely possible that cardiac fibroblasts can adopt more than one phenotype in response to injury.

Along those lines, while in our work we demonstrated that fibroblasts adopt an endothelial phenotype, we did not perform an exhaustive characterization of other potential phenotypes they may also adopt. As an example, we did not determine whether these cells adopt a lymphatic phenotype, which would be closely related to an endothelial phenotype. Again, the tools to perform the study exist and it would be interesting to determine whether such other changes occur.

<u>How does phenotypic conversion occur during MEndoT?</u>

Another issue that was not exhaustively addressed during this study was how fibroblasts adopt an endothelial fate. It has been previously shown that fibroblasts adopt a proto-myofibroblast fate prior to becoming full-fledged myofibroblasts[13]. Do cardiac fibroblasts adopt an intermediary state during MEndoT before becoming endothelial-like cells or do they transition directly from one fate to another?

If cardiac fibroblasts adopt an intermediate state, do they regress to a multi-potent precursor prior to redifferentiation? If that were the case, would it be possible that since a subset of endothelial cells in the heart are also derived from a Wt1 lineage[14,15] that post-injury fibroblasts revert and then adopt the endothelial phenotype along a different branch of

the same Wt1 lineage? To a very limited extent, we have preliminary data that suggest that fibroblasts that expressed Wt1 during development are capable of adopting an endothelial phenotype. It would be an interesting line of questioning to determine whether the transition was Wt1 lineage specific or not.

Another question that was not addressed during our research was about phenotypic reversal. We showed that when cardiac fibroblasts are exposed to cellular stress a subset will adopt an endothelial phenotype. However, we did not determine whether once the stress was reduced or eliminated whether the MEndoT cells would revert to fibroblasts. Given that the change is p53 mediated, it is possible that once the stress, and therefore the p53 signaling, is removed the cells could revert. This would be a difficult question to address *in vivo* partly because of the labeling strategy necessary and because during the scar maturation process most of the fibroblasts and endothelial cells propagated during granulation tissue formation undergo apoptosis[16].

How does p53 specifically regulate MEndoT?

We demonstrate, through gain and loss of function approaches, that MEndoT is mediated by p53. However, in our study of the phenomenon of MEndoT, we did not exhaustively study how p53 regulates the process. There are a few key questions that would help our understanding of p53 based regulation.

First, since p53 has been linked to the cellular stress response through the MAPK pathway, it would be interesting to determine what upstream regulators trigger p53 activity. As an example, could TGF-β, which factors prominently in the fibrotic response, activate p53 activity? Could p38 and JNK, which are directly upstream of p53, play a role? Each of these molecular targets has pharmacological activators and inhibitors akin to Pifithrin-α and RITA, respectively, that could be used to test potential interactions.

In addition to upstream regulation, the question can also be asked whether p53 acts directly as a transcription factor on endothelial gene targets or works through an intermediary. To date, we have preliminary data that favors the former hypothesis. Using Chromatin Immunoprecipitation (ChIP), our collaborators have shown that p53 is enriched in the promoter regions of several endothelial genes after serum starvation which suggests that p53 gene regulation is direct. Still, the direct regulation has not been conclusively proven.

Another potential source of p53 regulation is through post-translational modification. p53 has been described as having as many as 50 sites for post-translational modification[17]. Any one or a combination of sites may regulate the process of MEndoT. Existing tools such as mass spectrometry could be used to determine whether post-translational modification of p53 plays a role in MEndoT. Finding pertinent post-translational modifications could allow for mutational screens for gain and loss of function studies as well as allowing drug development for specific activation of MEndoT.

Upregulation of p53 has also been linked to exist from the cell cycle[18,19]. It would be interesting to determine whether the p53 activation that takes place during MEndoT pushes the cell towards senescence and drives them down a specific alternative lineage.

How do MEndoT cells interact with neighboring cells after injury?

Given that most fibroblasts and endothelial cells undergo apoptosis as the fibrotic scar matures[16], it leads us to question what the longer term fate of MEndoT cells are or if their function is served after a brief time. If that is the case, then in addition to contributing to the restoration of blood flow to the injured area, are there any other interactions between MEndoT cells and the neighboring cells at the injury border zone? We did not explore these interactions but, as described in Chapter 1, fibroblasts interact closely with macrophages,

endothelial cells and even cardiomyocytes. Are these other cell types affected by MEndoT cells?

We also observed that the number of endothelial cells at the site of injury was increased when MEndoT was increased. Could it be that MEndoT promotes the proliferation of endothelial cells or could the proliferation be explained as a by-product of RITA treatment? These are all interesting questions that could be addressed during the continued study of MEndoT.

Are there potential therapeutic applications of MEndoT in the treatment of acute ischemic cardiac injury in Humans?

The discovery of MEndoT is a novel finding with therapeutic implications in the treatment of ischemic cardiac disease. As described in Chapter 4, by improving vascularity at the site of injury the adverse effects of cardiac injury were effectively reduced. While several methods of improving vascularity after cardiac injury have been explored, many carry with them potential drawbacks. As an example, cellular reprogramming of fibroblasts into endothelial cells using exogenous transcription factors carries the potential dangers of off-target reprogramming of other cells, cellular dis-regulation and tumor formation[20,21]. Another example, using the explantation, expansion in culture, and reintroduction of cardiac stem or vascular progenitor cells is somewhat invasive, time-consuming, and inefficient[22,23]. Manipulating MEndoT represents a potential step forward in neovascularization targeted treatment of ischemic cardiac injury without some of the drawbacks associated with other models. Specifically, RITA based treatment is non-invasive and can be administered readily after injury. Further, while there is potential for off-target effects, p53 targeted treatments like RITA are currently in clinical trials for the treatment of cancer[24]. Based on the outcome

of those studies, it may be found that RITA treatment has minimal off-target effects and could be a prime target for clinical studies.

Conclusions

As described in this dissertation, we have identified Mesenchymal-to-Endothelial Transition (MEndoT) as a previously undescribed phenomenon in the progression of wound healing after acute ischemic cardiac injury. Our findings demonstrate that cardiac fibroblasts contribute to neovascularization at the site of injury and that manipulation of MEndoT is a potentially novel therapeutic in the treatment of myocardial infarction. Moreover, our findings increase our understanding of the roles of fibroblasts in guiding the progression of wound healing and scarring and suggest that further study of the process may also contribute to the broader understanding of cellular plasticity and its regulation.

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